Scientific Proceedings
Sixth Symposium of the Section of Experimental Cancer Research (SEK) of the German Cancer Society

Sektion Experimentelle Krebsforschung (SEK) der Deutschen Krebsgesellschaft e.V.

Sixth SEK-Symposium

Heidelberg, 10–12 April, 1991

432 J Cancer Res Clin Oncol ISSN 0171-5216 JCROD7 117 (Suppl) S1–S60 (1991)
017152169100034H

B 1

EVIDENCE FOR ACTIVATION OF PHOSPHOLIPASE D INDUCED IN INTACT CELLS BY EPIDERMAL GROWTH FACTOR

Marietta Kaszkin, Volker Kinzel
Department of Pathochemistry, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg

Signal transduction induced by epidermal growth factor (EGF) via its receptor involves two second messengers, diacylglycerol (DG) and inositol trisphosphate, both products of phosphatidylinositol-specific phospholipase C which seem to participate in the regulation of cellular proliferation by EGF We observed that A431 cells and HeLa cells in response to EGF accumulate substantial amounts of phosphatidic acid (PA), the potential product of DG kinase However, PA appears earlier and stays longer elevated than expected from DG availability thus raising the possibility of an EGF-induced activation of a phospholipase D (PLD) In the presence of primary alcohols PLD effects the phosphatidyl transfer reaction producing PA-alcohol, a reaction known to be exclusively mediated by PLD in intact cells A431 cells and HeLa cells prelabeled with [^{14}C]-oleate produce in the presence of ethanol (0 5%) or 1-butanol (0 2%) substantial amounts of labeled PA-alcohol in response to EGF These results indicate that activation of PLD had occurred Activation of PLD may represent a novel signal transducing pathway which may participate in the regulation of cell proliferation by EGF - a hypothesis presently investigated in this laboratory

Supported by the Deutsche Forschungsgemeinschaft.

B 2

EFFECT OF HA-RAS ON BOMBESIN-INDUCED OPENING OF K$^+$-CHANNELS

Karl Maly, Irina Bichler*, Florian Lang* and Hans H.Grunicke
Institute of Medical Chemistry and Biochemistry and the *Institute of Physiology, University of Innsbruck, Innsbruck, Austria

Addition of bombesin to quiescent NIH 3T3 fibroblasts transfected with a glucocorticoid-inducible Ha-ras construct, elicits persistent oscillating hyperpolarisations of the plasma membrane attributable to the activation of Ca^{2+}-dependent K^+ channels. In the absence of the inducer, bombesin evokes only single, transient hyperpolarisations. In contrast to the activation of K^+ channels, expression of Ha-ras attenuates bombesin-induced inositol trisphosphate formation and causes a reduction of the bombesin-mediated release of intracellular Ca^{2+}. The bombesin induced hyperpolarisation is insensitive to pertussis-toxin, whereas the activation of phosphoinositide hydrolysis by bombesin is inhibitable by the toxin. As the mitogenic activity of bombesin is enhanced in cells expressing the Ha-ras oncogene, the stimulation of the Ca^{2+} dependent K^+-channels seems to be related to the mitogenic signal transduction of bombesin.

B 3

CASEIN KINASE II ANTISENSE OLIGODEOXYNUCLEOTIDES INHIBIT MITOGENIC SIGNALING IN HUMAN FIBROBLASTS

R. Pepperkok*, R. Jakobi, P Lorenz, W Ansorge* and W Pyerin
Institute of Experimental Pathology, DKFZ, and *European Molecular Biology Laboratory, D-6900 Heidelberg, F R G

Mitogens such as the epidermal growth factor (EGF) or serum stimulate, when applied to cell cultures, transiently CK II activity of cytosol suggesting a role of CK II in mitogenic signaling The human CK II is a tetrameric enzyme (1) composed of two kinase-active subunits (α and α') and two regulatory subunits (ß). Subunits α and ß were sequenced recently (2,3), thus allowing to investigate the role of CK II in mitogenic processes by antisense oligodeoxynucleotide methodology We demonstrate that synthetic oligodeoxynucleotides complementary to mRNA of α and ß subunits strongly inhibit the serum and the EGF proliferation stimulation of quiescent primary human fibroblasts. When applied 2 h prior EGF, antisense-α and antisense-ß inhibited growth stimulation by 51% and 64%, respectively, and when applied prior serum, by 31% and 44%, respectively. Introduction of mutations into the oligodeoxynucleotides reduced their antiproliferative effect, and application of sense-oligodeoxynucleotides neutralized the effect demonstrating the high specificity of the antisense action. The effect was reversible, i.e., cells re-entered cell cycle after oligodeoxynucleotide removal. Thus the continuous availability of CK II subunits is essential for the mitogenic signaling by serum and EGF to occur Changes of CK II subunits at protein level are currently under investigation. References Pyerin et al. (1987) Biol. Chem. Hoppe-Seyler 368, 215-227, (2) Meisner et al. (1989) Biochemistry 28, 4072-4076, (3) Jakobi, Voss, Pyerin (1989) Eur J. Biochem. 183, 227-233

B 4

TUMOR PROMOTION AND PROTEIN KINASE C

P Borowski, M Harbers, A Ludwig, T Kischel, H Hilz
Institut für Physiologische Chemie, Universität Hamburg

TPA-induced promotion of initiated epidermal cells in mouse skin can be inhibited by benzamide (Bam) and analogs Bam effectively suppressed inflammation and accumulation of white blood cells in TPA-treated skin without affection of hyperproliferation, suggesting that its antipromotor effect came about by the elimination of granulocyte-derived reactive oxygen species However, Bam and analogs also suppressed TPA-induced promotion of "initiated" epidermal JB6 cells to the tumor phenotype (anchorage-independent growth tumor promotion in nude mice) in the absence of granulocytes indicating that mechanisms not involving reactive oxygen were responsible for promotion Since promotion in vivo and in vitro requires prolonged exposure to TPA, we considered reduced rather than enhanced activity of protein kinase C (PKC) as a signal for the induction of the tumor phenotype Evidence for such a mechanism was provided by a Western blot analyses of TPA-induced PKC depletion, by the promoting activity of certain PKC inhibitors in the absence of TPA and by the synergistic effect with TPA of PKC antisense oligonucleotides Promotion can also be effected by EGF and by TGF-α. Co-administration of Pertussis toxin eliminated induction of anchorage-independent growth by all these promoting substances A Gi-like protein, therefore, appears to be involved in the transmission of the signal leading to the tumor phenotype Partial amino acid sequence of Gi ADP-ribosylated *in vivo* as well as immunological data indicate that the PTX-sensitive acceptor is a α-2 containing Gi subspecies. A possible link between PKC, active Gi protein, and tumor promotion will be discussed

B 5

MONOCLONAL AND POLYCLONAL ANTIBODIES AGAINST CASEIN KINASE II SUBUNITS

P.Lorenz, A Wedell, R.Jakobi, W.Pyerin

Institute of Experimental Pathology, DKFZ, D-6900 Heidelberg, F.R.G

Antibodies represent sensitive and effective tools to investigate structure and function of proteins The Casein kinase II (CKII) is composed of two catalytic (α and α') and two presumably regulatory subunits (β) These polypeptides show markedly conserved amino acid sequences explaining the failure to generate antibodies with the purified enzyme by routine strategies Our approach therefore was to express the CKII subunits as fusion proteins in E. coli using cDNA-clones coding for human CKII-α and -β subunits, respectively, developed in our laboratory, and a pEX31-derived expression-vector (Strebel et al. (1986) J Virol **57** , 983-991) Three different fusion proteins, linking the polypeptides to the N-terminal 10 5 kD-fragment of the MS2 RNA-polymerase β-chain, were expressed under control of the temperature inducible λPL promotor; αFus54 containing the whole CKIIα-chain, αFus26 containing amino acid sequence 1-128 of α-subunit, and βFus32 containing amino acid sequence 20-200 of β-subunit. Purification of fusion proteins was facilitated by their insolubility in 1M urea in contrast to the bulk of bacterial proteins Crude sera obtained after one priming and three booster injections into rabbits displayed high reactivity with human CKII holoenzyme (ELISA) and the respective subunits (Western blotting) For monoclonal antibody production, hybridomas were generated by fusing spleen cells of a mouse hyper-immunized with βFus32 and mouse Ag8 653 myeloma cells Four clones secreting specific monoclonal antibodies reacting with the CKII holoenzyme were identified by ELISA. They were grown in serum-free medium and the antibodies purified by chromatography on protein A- or protein G-sepharose Three of them were able to detect β protein on Western blots, even in crude cell extracts Two of them were classified as IgG1, one as IgG2b and the fourth as IgG3, all of them possessing κ light chains

B 6

RELATIONSHIP BETWEEN 43 KDa EPIDERMAL GROWTH FACTOR-RELATED CLONOGENIC ACTIVITY AND CLINICAL PARAMETERS FOR BREAST-CANCER

K. Eckert, A. Granetzny, J. Fischer, and R. Grosse, Central Institute of Molecular Biology, (K.E., R.G.), Central Institute of Cancer Res. (A.G., J.F.), Berlin-Buch

The clonogenic activity of a previously purified 43 kDa EGF-related protein was estimated in a Bio-Gel P-30 fraction obtained from the urine of breast cancer patients. Increase of activity was statistically significant in lymph node-positive patients, in a group of patients with larger carcinomas, with accelerated tumor growth, in premenopausal patients and younger age and in estrogen receptor negative patients. In 31 patients the activity was estimated during polychemotherapy before surgery. Differences between the values at the begin and the end of therapy were compared between all groups be the Kruskal-Wallis test ($p=0.02$). Patients with progressive disease showed increasing activities (mean values from 315 to 811) while in those with complete remission activity decreased (from 449 to 213). Partial- and non responders showed no change. In a long-term follow-up study with 25 patients the pre- and postoperative activities were estimated. In 17 of 18 patients who had no local recurrence or metastasis the activity declined after surgery, whereas in 6 of 7 patients who died, activity increased 2-5 fold prior death. A life table analysis with a total of 101 patients revealed a trend to shorter survival in the group with higher activity ($p=0.042$). These observations suggest a role for the EGF-like growth factor activity in breast cancer diagnosis and prognosis.

B 7

GLUCOCORTICOID RECEPTOR DETERMINATION IN ACUTE CHILDHOOD LEUKEMIA WITH REGARD TO THEIR CLINICAL RESPONSE TO STEROID THERAPY

V Mauch, S Wehner, B Kornhuber and U Ebener
Clinic of Pediatrics, Dept. of Hematology and Oncology, J W Goethe Univ , Theodor-Stern-Kai 7, D-6000 Frankfurt/M 70, Germany

The determination of the GCR-level in acute leukemias still play an important role in evaluating the clinical response of leukemias to steroid therapy. In addition to other factors influencing the response to therapy, it seems to offer a clue to the prognosis of the different types of leukemias

In our study we concentrated on the investigation of these biochemical markers in leukemic blasts in children (n=16) after phenotyping with an immunoenzymatic staining (APAAP-complex-technique) The mononuclear cells (MNC) of isolated bone marrow aspirates (BM) were analysed using a large panel of monoclonal antibodies (MoAbs) in order to determine the lineage of the leukemic blasts as well as their stage of differentiation. According to the results a classification was carried out (n=16, 8 cALL, 4 AML, 3 T-ALL, 1 AUL) The GCR-level was measured by a whole cell-binding-assay using [3H]-Dexamethasone (DX) In each experiment the leukemic cells were incubated either with radiolabelled DX alone or in the present of a greater than 100 fold excess of unlabelled DX in order to determine the specific binding of the tracer In addition to freshly from bone marrow prepared leukemic cells we also investigated the leukemic cell line Molt 4 for their receptor level as well as peripheral blood lymphocytes from healthy donors The number of receptor sites was estimated mainly by scatchard plot analysis In some experiments, due to the limited cell numbers, comparisons were made by determining receptor binding at a single concentration (near maximum) of [3H]-DX

The results to-date point into the direction that among the group of ALL the receptor level ranged from 2 800 - 11.300 sites/cell (specific binding between 21% and 67%) The GCR-level of the AML probes showed a low specific binding ranging from 2 100 to 8 400 sites/cell (percentage of specific binding between 15% and 60%) The receptor level of the cell line Molt 4 averages around 50 3%, which amounts to a receptor level of 8.600 sites/cell.

All results gathered from our experiments so far are in favour of a correlation between the GCR-level and the response to GCR-therapy although among the cases of ALL 2 cases showed deviating results

B 8

DEVELOPMENT OF AN IN VITRO IRRITANCY AS AN ALTERNATIVE TO THE ANIMAL DRAIZE TEST

K. Müller-Decker, G. Fürstenberger, I. Vogt and F Marks
Institute of Biochemistry, German Cancer Research Center,
Im Neuenheimer Feld 280, FRG-6900 Heidelberg

In acute toxicity screening the rabbit eye irritation test introduced by Draize is the method currently used to evaluate the irritant potential of chemicals designed for industrial pharmaceutical and cosmetic use .Due to the lack of validated alternatives, this test is still in use despite the scientific and ethical criticisms and the high costs Recently, various methods have been developed with the goal to replace partially or wholly the animal use in irritation testing In this study a mechanistic approach is currently initiated to develop an alternative in vitro method. The strategy is based on the steadily increasing body of evidence indicating that chemical and physical skin irritants, proinflammatory mediators and wound hormones although following different mechanistic routes in epidermal cells induce the release of arachidonic acid (AA) and their metabolism into highly active biomolecules along the cyclooxygenase and lipoxygenase pathway Thus, arachidonic acid release and prostanoid formation in keratinocyte cells appear to be promising candidates for in vitro parameters of irritancy To find a suitable test culture, skin derived primary and secondary human keratinocytes which are assumed to be the primary target for irritants in epidermis were compared with respect to their capability to produce eicosanoids after challenging with irritants or proinflammatory mediators.

Initial studies revealed that the profile of eicosanoids released upon treatment was unique for each cell type tested. In all cell types used, the release of arachidonic acid was stimulated by such as the irritant Ca^{++}ionophore A23187, the PKC activator TPA or the peptide bradykinin within a few minutes. With respect to prostanoid synthesis qualitative differences between the cell types were noted. While in primary NHEK cells and in the HPKII line a parallel increase of both AA and PGE_2 was measured when the cells were treated with bradykinin or A23187 in the HACAT cell line a 2-4 hour delay of PGE_2 synthesis as compared with AA-release increasing immediately after begin of treatment was observed. Moreover, in HACAT cells TPA-induced prostanoid release was found to be inhibited by actinomycin D and cycloheximide indicating transcriptional and translational control of PGH-synthase These results suggest that there is a correlation between exposure to irritants and eicosanoid release in human HPKII and NHEK keratinocytes. These cells may prove therefore, to be suitable as animal free test system for dermal irritants

B 9

LASER PHOTODYNAMIC THERAPY (PDT) AND PHOSPHOLIPID METABOLISM IN HUMAN TUMOR CELLS - 202 MHz ^{31}P-NMR INVESTIGATIONS

G Graschew, A Küsel, W Hull, W. Lorenz, H. W Thielmann,
German Cancer Research Center, D-6900 Heidelberg, FRG

Aim To increase our basic understanding of ^{31}P-NMR spectra obtained at low field from tumors in patients and to interprete the changes occurring during tumor growth and therapy we investigated (^{31}P-NMR spectra) human tumor xenografts in nude mice at high field and determined quantitatively several phospholipid metabolites· nucleosidedi- and -triphosphates (NDP and NTP), phosphocreatine (PCr) and cytidinediphosphocholine (CDPC)

Techniques: Cells of various human carcinomas (CX1 colon, LX1 lung, MX1 mamma) were cultured in basement membrane gel For NMR measurements $2x10^7$ living cells embedded in gel were placed in a home-built sample chamber and perfused at 1-2 ml/min with HAM's F-12 medium containing in addition 10% fetal calf serum and 20 mM HEPES (pH 7 4) After 2 h incubation with PEOM-photosensitizer (5 µg/ml) samples were irradiated with laser light (wavelength. 630 nm, dose rate 80 mW/cm², dose 20 J/cm²)

Results The metabolic response of tumor cells to PDT was examined After 4-6 h, the intracellular pH decreased from 7 2 to 6 8 The total cellular phosphate detected decreased by 25-30% (shedding of damaged cells from the gel threads) NTP and NDP decreased from 25-30% to 10-15%, PCr levels from about 5-8% to 1-2% of total phosphate A signal from the β-phosphate of CDPC at -8.98 ppm was detected CDPC level was 5-6% of total phosphate 30 h after PDT, the intracellular pH was identical with the external pH and the total cellular phosphate detected had decreased by 60-70% Only a small number of vital cells (3-5%) was observed after PDT in the gel threads

Conclusions 1 Using ^{31}P-NMR, it is possible to follow the efficiency of laser photodynamic therapy (PDT) of human tumors in a non-invasive way
2 After PDT, an increasing CDPC signal was observed and the total cellular phosphate detected decreased indicating a high shedding of damaged cells from the gel threads
3 Quantitation and determination of the kinetics of CDPC accumulation in the ^{31}P-NMR spectra of human tumors can be used as criteria for the efficiency of the PDT

B 10

COVALENT BINDING TO PROTEINS OF ARACHIDONIC ACID METABOLITES FORMED BY PROSTAGLANDIN H SYNTHASE

Gisela H Degen and Alexius Freyberger
Inst. für Toxikologie der Universität Wuerzburg
Sonderforschungsbereich 172

Prostaglandin H synthase (PHS) converts arachidonic acid (ARA) to prostanoids which have been implicated as important mediators in tumor promotion and progression. Interestingly, when microsomal PHS is incubated with radiolabeled ARA, a non-extractable fraction of the radioactivity precipitates with proteins indicating formation of reactive fatty acid intermediates/metabolites. Protein binding was further studied in incubations with labeled ARA and purified PHS-holoenzyme and analyzed by SDS-polyacrylamide gel electrophoresis. radioactivity determined in the separated protein bands indicated covalent binding of reactive ARA metabolite(s). Incubations carried out in the presence of albumin (BSA) resulted in binding not only to PHS but also to BSA; likewise other proteins (globulin, ovalbumin, actin) could be covalently modified by ARA-metabolites *in vitro*. The unstable prostaglandin (PG) intermediates PGG_2 and PGH_2 are both considered as candidates for protein alkylation although this awaits final confirmation.
Our results show that PHS-dependent oxidation of ARA is accompanied by the formation of fatty acid metabolites which bind covalently to proteins. Further investigation *in vivo* regarding the patho-/physiological significance of this protein modification is required.

This work has been supported by a grant from the Deutsche Forschungsgemeinschaft.

B 11

ADDUCTS WITH HUMAN BLOOD MACROMOLECULES AS BIOMONITOR FOR EXPOSURE TO SUSPECTED CARCINOGENS e.g 1,2-EPOXYBUTENE-3, THE REACTIVE INTERMEDIATE OF 1,3-BUTADIENE

A. Muller, M Linscheid*, Ulrike Hindermeier, Ute Jorritsma, K Golka, W Föllmann, H. Peter, H.M Bolt

Exposure to suspected carcinogens is usually monitored by measuring the concentration in ambient air or by quantification of the substance or its metabolites in blood or urine The genotoxic effect can however not be monitored by these methods, since interindividual differences in metabolism and stereochemical effects influence the biological effective dose Binding products of alkylating agents with DNA in lymphocytes, or as surrogate with blood proteins, reflect the individual health risk much better than measuring the parameters mentioned above In pharmacology, albumin "binding" of pharmaceutics is well investigated, an implementation for exposure monitoring in toxicology is however not yet established This study presents initial experimental results for the use of adducts of 1,2-epoxybutene-3 with human blood macromolecules as biomonitors which include the effects of individual variations of the exposure to 1,3-butadiene

The reactive 1,2-epoxybutene-3 was added to a 5 ml aqueous solution of 10 mg/ml human hemoglobin or human serum albumin and incubated for four hours in 22 ml head space vials under constant rolling at 37°C, then shock frozen in liquid nitrogen and lyophilized to remove unreacted epoxide. The proteins were redissolved in 5 ml buffer (10mmol Tris, 100 mmol sodium acetate, 0.1 mmol $CaCl_2$, pH 7 4) and 0.5 ml aliquots were drawn for heat denaturation of proteins (95°C for 30 min and subsequent cooling in ice water prior to tryptic or proteinase K digestion with subsequent peptide mapping through hplc analysis on a RP 18 column)

The alkylated amino acids within the peptides were then characterized by an hplc-mass spectrometry analysis Hplc was performed on a RP 18 microbore column with methanol/H_2O/1% trifluoroacetic acid and glycerine for elution. Fast Atom Bombardment (FAB) mass spectrometry analysis was done on a MAT 90 spectrometer (Finnigan, Bremen) Alkylation products in human blood proteins were determined by both methods peptide mapping and mass spectrometry following hplc These methods are therefore suited for further development as *in vivo* monitors for occupational exposure to 1,3-butadiene, of which 1,2-epoxybutene-3 is the main biologically reactive and genotoxic metabolite

Institut für Arbeitsphysiologie, Ardeystr 67, W-4600 Dortmund 1
*Institut für Spektrochemie, Bunsen-Kirchhoff-Str 2, W-4600 Dortmund 1

B 12

Adduct patterns detected by ^{32}P-postlabelling of 1-hydroxymethylpyrene sulfate and 1-chloromethylpyrene in *Salmonella typhimurium* and with free salmon sperm DNA

S Honnerjahn, B H Phillips[1], A Hever[1], A Seidel, H R. Glatt
Institute of Toxicology, University,D-6500 Mainz, FRG and [1] Haddow Laboratories,Belmont, Surrey SM2 5NG, UK

1-Chloromethylpyrene (CMP), formed from 1-hydroxymethylpyrene sulfate (SMP) in the presence of Cl^- in physiological concentrations, is much more mutagenic in *Salmonella typhimurium* TA98 than is SMP (1) Assuming that DNA adducts are the cause of these mutations, differences either in the access to the DNA or in intrinsic reactivity towards DNA could account for the difference in mutagenic activity To distinguish between these two possibilities, we have now incubated CMP and SMP with bacteria and free salmon sperm DNA in the presence and absence of Cl^- ions, and analyzed the quantity and the pattern of DNA adducts
Under all these conditions adducts were formed, the chromatographic patterns containing the same four major and several minor adduct spots Incubations with CMP and SMP, in the presence and absence of Cl^-, resulted in virtually identical adduct patterns, but variable adduct levels. In bacteria, the adduct levels, like the mutagenic activity, followed the order $CMP/Cl^- > CMP/H_2O \cong SMP/Cl^- >> SMP/H_2O$ In reactions with free DNA, the relative intensity of the major spots and the number of minor spots differed from the results obtained in bacteria, possibly due to the different A/G ratios of bacterial and vertebrate DNA SMP and CMP formed very similar adduct patterns, the quantitative difference being much smaller than in the experiments in bacteria. Moreover, the presence of Cl^- had only a weak influence on the binding of SMP These results show that SMP and CMP have qualitatively and quantitatively similar intrinsic reactivities towards DNA Hence, the different biological activity in bacteria is attributable to differential access of the compounds to the target DNA Whereas CMP is highly lipophilic, SMP is ionized at physiological pH values and therefore may have difficulties in penetrating cell membranes

1) H.R Glatt, H. Hencshler, D H Phillips, J M Blake, P Steinberg, A. Seidel, F. Oesch, Environ Health Perspect., 88 (1990) 43 - 48

B 13

GLUCURONIDATION OF N-NITROSO-METHYLPENTYLAMINE IN RATS

K Wiench, E Frei, P Schroth, M Wiessler
Institut für Toxikologie und Chemotherapie
Deutsches Krebsforschungszentrum, Heidelberg

Besides an activation to alkylating species, there is also a detoxification pathway for N-nitrosodialkylamines by glucuronidation The balance between UDP-glucuronosyltransferase (UDPG) and ß-glucuronidase may determine the amount of glucuronide found in a certain organ

In vivo and in vitro experiments were carried out with 14C-labelled N-nitrosomethylpentyamine (NMPentA) and both possible 1-glucuronides were synthesized as marker compounds

In hepatocytes from untreated rats 1 2% of 15uM NMPentA were conjugated to the glucuronide in the methyl-group Treatment of rats with phenobarbital inhibited 1-C-hydroxylation of NMPentA, nevertheless 1 6% of 15uM NMPentA were conjugated to glucuronide by these hepatocytes, phenobarbital therefore induces a UDPG In hepatocytes of 3-methylcholanthrene and arochlor pretreated rats the 1-glucuronide in the pentyl-chain was also formed 3-Methylcholanthrene pretreatment lead to 0 042% pentyl- and 0 85% methyl-glucuronide, while arochlor lead to 0 08% pentyl- and 1 1% methyl-glucuronide, if the respective hepatocytes were incubated with 15uM NMPentA. No glucuronides were formed in incubations of kidney cells with NMPentA In vivo 2 86% of 1 56 mg / kg rat NMPentA were found in urine as glucuronide in the methyl-group The glucuronidation of NMPentA is therefore regioselective

B 14

TOXICOKINETICS AND TOXICODYNAMICS OF 15,16-DIHYDROSIMPLEXIN AS A REPRESENTATIVE DITERPENE ESTER OF THE DAPHNANE TYPE TUMOR PROMOTORS

T Schäfer, M.Hergenhahn and E Hecker
Inst. für Biochemie DKFZ Heidelberg

To study the toxicokinetics and toxicodynamics in vivo, a new radioactive labeled derivative of simplexin, the prototype of the daphnane type orthoesters, 15,16-dihydrosimplexin (DHS) was synthesized by partial hydrogenation of Excoecaria factor OP_2 with tritium gas 1h after epicutaneous exposure of NMRI-mice to 10 nmoles of radioactive DHS the activity in the surface lipids was 39 %, after 6h decreasing to 7,5 % and then remaining constant up to 72h During the first 24h the radioactivity in the epidermal and the dermal fraction was 8 % and 7 % respectively of the applied compound, decreasing to 1 % up to 72h; in the subcutis activity was below 0,3 % of dose These facts show that only a small quantity of radioactivity was transported to blood via skin After a lag time of 1h radioactivity appeared in the stomach, presumably because mice lick their back skin and incorporate the diterpene ester The intestine passage was very quick - after 12h 60 % of the activity was detected in the feces After 72h, approximately 90 % of the applied dose were detected in feces and urine Up to 6h the level in liver remained constant (2-3 %), decreasing to 0,2 % after 72h

Metabolites were identified by HPLC and enzymatic reactions. In skin, DHS underwent no metabolic alteration The major metabolic pathway started in liver through opening the oxirane ring by an epoxide hydrolase, yielding the 6,7-diol The orthoester and its 6,7-diol were glucuronated to give a mixture of various 5-, 20-, 6- and 7-glucuronides, which were found in liver and intestine, likewise 20-acyl-compounds were detected in intestine, but not in excrements. In feces DHS and glucuronides and in urine only the glucuronides were found In general, the orthoester function was not attacked

Thus, the diterpene ester type promoters of the daphnane series are not activated by biotransformation None of the metabolites bind to the mouse brain membrane fraction, containg Protein Kinase C, suggesting that daphnane type esters themselves are the active principles ("ultimate promoters"), responsible for the pleiotropic biochemical reactions observed

B 15

Collagen Neosynthesis In Cultured Human Peritumorous Tissue Is Enhanced By Viscum Album Extract

D Proft, P. Bartholmes

Inst für Biochemie der Universität Witten/Herdecke

Penetration of basement membrane and infiltration of interstitial stroma by invasive tumors requires lysis of constituents of extracellular matrix e g collagen (1). Clinical evidence exists, that encapsulation of tumor and reduced metastasis may be due to the action of Viscum album extracts in peritumorous tissue With respect to other tumor agents this effect has been demonstrated using histological methods (2) The aim of this study was to quantify the rate of neosynthesis of collagen by fibroblasts in peritumorous connective tissue as a marker for encapsulation Furthermore the activity of tumor related collagenases - which play a critical role in metastatic processes was investigated Tissue culture was chosen as in vitro system with most physiological relevance. Extraction and Quantification of collagen in small tissue cultures was developed A therapeutical concentration (0 04% (v/v)) of aqueous extract of Viscum album L.,ssp. mali (Helixor®) enhanced the rate of collagen neosynthesis by about 43% as measured via incorporation of ^{14}C-proline into newly synthesized collagen during tissue culture. On the other hand a 30% reduction of tumor related collagenases activity was measured Significant differences have been found for human mamma- and colon-carcinoma, respectively, taken from 14 patients. Biochemical data were correlated with histological characteristics.

(1) L.A Liotta, C N. Rao, S.H Barsky, Lab. Invest 49 636, 1983
(2) T. Kimoto, Cancer Detection and Prevention 5 301, 1982

B 16

MODIFYING EFFECTS OF DITHIOCARBAMATES ON THE INHIBITORY ACTIVITY OF PROTEIN SYNTHESIS BY N-METHYL-N'-NITRO-N-NITROGUANIDINE (MNNG)

R.S Bagewadikar, B.Bertram, N.Frank and M Wiessler
Institute of Toxicology and Chemotherapy, German Cancer Research Center, Heidelberg, Germany

The influence of dithiocarbamates on the N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induced inhibition of cell free protein synthesis was investigated. Prolinedithiocarbamate (PDTC) and diethyldithiocarbamate (DDTC) were added to a protein synthesizing system using postmitochondrial supernatant (S-30 fraction) or a defined system consisting of purified polysomes and 'pH5' enzyme fraction to observe their effect on MNNG induced inhibition of amino acid incorporation into polypeptides.

PDTC showed a concentration dependent increase of MNNG induced inhibition of amino acid incorporation, while DDTC decreases this inhibition in S-30 fraction. However, the modifying effect of PDTC and DDTC showed a different picture in a protein synthesizing system consisting of purified polysomes Here PDTC at increasing concentrations did not alter the MNNG induced inhibition of amino acid incorporation while DDTC showed a partial reduction in the inhibitory activity of MNNG When cytosol (S100) fraction was added to the system the MNNG induced inhibition was reversed by both of these dithiocarbamates. Thus PDTC and DDTC show partial modulation against inhibitory activity of cell free protein synthesis by MNNG.

B 17

A CALCIUM-UNRESPONSIVE PROTEIN KINASE C OF THE δTYPE

Hanno Leibersperger, Michael Gschwendt and Friedrich Marks
Institut für Biochemie, Deutsches Krebsforschungszentrum Heidelberg

A calcium-unresponsive protein kinase (PKC) was purified to homogeneity from porcine spleen. A characterization of this enzyme will be presented. The novel kinase is recognized by an antiserum raised in rabbits against a peptide of the COOH-terminus of δPKC. It is neither recognized by an α,β,γPKC-specific nor bei an εPKC-specific antiserum. The δPKC-specific antiserum reacts also with respective kinases of other species. The apparent molecular weights of these kinases differ slightly. An 82 kDa kinase (p82-kinase) is found in several murine tissues, predominantly in brain, lung, kidney, uterus, placenta, and epidermis. Whereas α,β,γPKC is about equally distributed between the particulate and the soluble fraction of each tissue, the δPKC-like p82-kinase is almost exclusively located in the particulate fraction. Murine epidermis contains much more p82-kinase than α,β,γPKC. This is of special interest with respect to the phorbol ester-induced tumor promotion in mouse skin. Topical application of the phorbol ester TPA to mouse skin causes down-regulation of the epidermal δPKC-like p82-kinase, as determined by immunoblotting. 18 hours after TPA more than 80% of the enzyme is degraded and the recovery is very slow.

B 18

PHOSPHORYLATION OF GLUTATHIONE S-TRANSFERASE BY PROTEINKINASE C

S.Fasco[1], E.Frei[1], W.Pyerin[2]
[1] Institut für Toxikologie und Chemotherapie
[2] Institut für Experimentelle Pathologie
Deutsches Krebsforschungszentrum, Heidelberg

Glutathione S-Transferases (GST) play an important role in the metabolism of xenobiotics. They have three different functions: Conjugation with glutathione and peroxidase activity, reversible binding of hydrophobic compounds and covalent binding to reactive electrophiles. Taniguchi and Pyerin (1989, Bioch.Bioph.Res.Com. 162, 903) succeeded in phosphorylating three GST isoenzymes by proteinkinase C (PKC) and found a decreased affinity of transferase 1-1 for bilirubin.
Now the effects of phosphorylation of GST by PKC on the transferase- and peroxidase-activity were examined. PKC was partially purified from rat brain. GST from rat liver (purchased from Sigma) moved as three bands on SDS/polyacrylamid-gelelectrophoresis all of which were phosphorylated. About 15% of the GST molecules incubated with PKC were phoshorylated within 45 minutes . The transferase activity (expressed as nmol/min/µg) with 1-Chloro-2,4-dinitrobenzene (CDNB) was 26.4 ± 1 and after phosphorylation it was 26.3 ± 0.1. Dephosphorylation of GST with alkaline phosphatase did not change the activity with CDNB either. The peroxidase activity with cumenhydroperoxide was 6.3 ± 0.2 and after phosphorylation 5.9 ± 0.1. The data indicate that in vitro both catalytic activities of GST are not regulated by phosphorylation through PKC.

B 19

HELA CELLS TREATED WITH CALCIUM IONOPHORE A23187 ACCUMULATE ESTER- AND ETHER-LINKED PHOSPHATIDATES THROUGH ACTIVATION OF PHOSPHOLIPASE D

Peter Plein, Marietta Kaszkin, Volker Kinzel
Department of Pathochemistry, German Cancer Research Center,
Im Neuenheimer Feld 280, D-6900 Heidelberg

Metabolism of phospholipids is crucial to cellular activation. Agonist-induced accumulation of phosphatidic acid (PA) species gains increasing attention since PA does not simply represent a source of diacylglycerol, the activator of protein kinase C, but an effector of its own in processes such as control of cellular proliferation, cell cycle, secretion, etc. We tested the possibility of an involvement of phospholipase D (PLD) in the response of HeLa cells to calcium ionophore A23187. HeLa cells prelabeled with [^{14}C]arachidonic acid respond to ionophore A23187 with a rapid accumulation of comparable amounts of labeled 1,2 acyl-phosphatidic acid (acyl-PA) and 1-0-alkyl-2 acyl PA (alkyl-PA) with a first peak at 5 min and a second increase starting at 20-30 min. In cells prelabeled with [^{14}C]oleic acid the ionophore mobilizes relatively more of labeled acyl-PA. The generation of labeled acyl-PA in cells prelabeled with [^{14}C]palmitoyl-lysophosphatidylcholine indicates the derivation of PA from phosphatidylcholine. The transphosphatidylation of labeled acyl- and alkyl-PA to 1-butanol in all cases shows that activation of phospholipase D had occured. The reaction became detectable at 10^{-6} M ionophore and was fully expressed at 10^{-5} M. PA-butanol generated during 1 h treatment with ionophore amounted to approx. 0.5 nmol per 10^6 cells (i.e. 10^{-4} M conc. within cells) as shown by the use of [^{14}C]-n butanol. The data demonstrate that HeLa cells accumulate amounts of PA sufficient to alter membrane properties.
Supported by the DFG.

B 20

DIFFERENT EFFECTS OF EXOGENOUS PI- AND PC-SPECIFIC PHOSPHOLIPASES C ON PHOSPHOLIPID METABOLISM OF INTACT HELA CELLS

Karin Schiess, Marietta Kaszkin, Lothar Seidler and Volker Kinzel
Department of Pathochemistry, German Cancer Research Center,
Im Neuenheimer Feld 280, D-6900 Heidelberg

Agonists such as growth factors induce a cascade of changes in cellular phospholipid metabolism to yield inter- and intracellular messengers for cellular stimulation. Diacylglycerol (DG) mostly derived from phosphatidylinositol (PI) or phosphatidylcholine (PC) through the action of specific phospholipases C (PLC) plays a key role in the activation of protein kinase C (PKC). In order to focus attention to consequences of activation of a particular PLC, intact HeLa cells were treated with the exo-genous PC-PLC (from Cl. perfringens) or PI-PLC (from B. cereus). Both enzymes rapidly mobilize DG which is known to undergo a rapid trans-bilayer flip. Only PC-PLC led to morphological changes which were reversible on enzyme removal. In cells prelabeld with radioactive arachidonic acid (AA) labeled DG became mobilized. Only PC-PLC induced the release of AA with a lag of >10 min. AA release requires a threshold dose of PC-PLC and a minimum time of treatment beyond which a AA release appears to be independent of the further presence of PC-PLC. AA release occurred also after downregulation of PKC by TPA. Fluocinolone acetonid, an inhibitor of TPA-induced AA release, did not influence PC-PLC induced AA release thus indicating that phospholipase A2 was probably not responsible. The level of monoglycerids appeared to be elevated, a fact which may point to activation of a DG lipase. It may be possible that different DG species derived from PI and PC may contribute to the observed differences in the biological activity of both PLC.
Supported by the Deutsche Forschungsgemeinschaft.

B 21

CHARACTERIZATION OF 8-LIPOXYGENASE ACTIVITY INDUCED BY TPA IN MOUSE SKIN IN VIVO

T. Jacobi, G. Fürstenberger, E. Besemfelder, M. Stephan*, W. D. Lehmann* and F. Marks, Department of Biochemistry and *Central Spectroscopy Unit , German Cancer Research Center; D-6900 Heidelberg, F.R.G.

Dioxygenase-catalyzed peroxidation of lipids is a phenomenon that leads to highly tissue-specific distribution patterns of biologically active hydroperoxides and hydroxides. The epithelial compartment of murine skin has been shown to contain a 8-lipoxygenase activity only after treatment with the tumor promoter TPA (tetradecanoylphorbol acetate). This activity is the result of a de-novo synthesis which can be prevented by cycloheximide. The induction of 8-lipoxygenase during the phorbol ester-triggered hyperplastic transformation of the epidermis is interpretable as a gene activation that is mediated by protein kinase C.
Inhibition studies in cell-free systems have shown this enzyme to be a member of the lipoxygenase family. The primary product formed from arachidonic acid is 8-HPETE (hydroperoxyeicosatetraenoic acid) and the enzyme appears not to possess a significant peroxidase activity since 8-HPETE can be isolated from the reaction mixture. C_{18}-fatty acids like linoleic and linolenic acid are also accepted as substrates, but the turnover is lower than with arachidonic acid. The role of phospholipids as putative substrates is under investigation.
8-HPETE/8-HETE levels in vivo are dramatically increased after phorbol ester treatment - in agreement with the induction of 8-lipoxygenase observed, which raises the question of the immediate biochemical consequences. Preliminary results suggest a clastogenic effect of such oxidized eicosanoids. In spite of being a cytosolic enzyme 8-lipoxygenase appears to possess a lipid binding site and is activated by lecithin. The enzyme does not require calcium ions or ATP and shows a pH optimum at 7.5. The enzyme has been purified to near homogeneity and a peptide-derived antibody has been obtained. Oligonucleotides representing conserved regions in DNA sequences of cloned lipoxygenases are used to screen cDNA libraries obtained from phorbol ester-treated mouse epidermis with the intention to isolate the gene of 8-lipoxygenase.

B 22

HETEROGENEITY OF TNF/LT RECEPTORS ON ACTIVATED T CELLS

M. Grell, B. Thoma, P.Scheurich.
Klinische Arbeitsgruppe der MPG, Göttingen

Both tumor necrosis factor (TNF) and lymphotoxin (LT) act as costimulators for activated normal human T lymphocytes. Recent studies have shown that two distinct TNF/LT receptors molecules (p60 and p80) are coexpressed on neoplastic cell lines. Cross-competition studies on activated human T cells with TNF and LT indicate that both cytokines bind to one receptor type. In addition, Scatchard analyses of saturation binding experiments with ^{125}I-TNF and ^{125}I-LT revealed only one class of high affinity binding sites. When the radiolabeled cytokines were crosslinked to the binding protein, in both cases a major 100 kDa complex was found, representing the p80 receptor attached to monomeric TNF or LT. However, by the use of a p60 specific antibody (H398) we could show for the first time that both receptors are coexpressed on T lymphocytes in an activation dependent manner. Though p60 receptors accounted for only 6% of total high affinity TNF/LT binding sites, blocking of ligand binding to p60 by H398 strongly antagonized typical TNF and LT responses on activated T cells. Furthermore, whereas both cytokines bound with high affinity to p80, we show here that the lower specific bioactiviy of LT, compared to TNF, correlates with its lower affinity to p60 receptors, indicating a potential causal relationship. Together, these results demonstrate that ligand binding to p60 and not to p80 is response limiting in T cells and underline the crucial role of p60 in TNF and LT signaling.

B 23

STIMULATION OF K^+-TRANSPORT SYSTEMS BY HA-RAS

Karl Maly, Markus Meyer and Hans Grunicke
Institute of Medical Chemistry and Biochemistry,
University of Innsbruck, A-6020 Innsbruck/Austria

The expression of Ha-ras in quiescent NIH3T3 cells carrying a glucocorticoid-inducible human Ha-ras gene (val-gly mutation at codon 12) stimulates total $^{86}Rb^+$ influx. This effect is predominantly due to an elevated $^{86}Rb^+$-uptake through an ouabain-resistant, furosemide-sensitive system. The ouabain-sensitive Na^+/K^+-ATPase is less affected. The transport which is resistant to both inhibitors is not altered by Ha-ras. Overexpression of the Ha-ras proto-oncogene causes only a marginal increase in total $^{86}Rb^+$-uptake. The stimulation of the furosemide-sensitive influx by Ha-ras is paralleled by an increase in mean cell volume which can be inhibited by furosemide. A rapid stimulation of the furosemide-sensitive Rb^+-influx is also observed after addition of bombesin to growth arrested cells. Furosemide inhibits the mitogenic response after expression of Ha-ras or addition of bombesin. Both, the Ha-ras as well as the bombesin-induced stimulation of the furosemide-sensitive Rb^+-transport can be blocked by protein kinase C-depletion or the protein kinase C inhibitor staurosporine. In contrast to bombesin-induced phosphatidylinositol-4,5-bisphosphate hydrolysis which is down- modulated by Ha-ras, the stimulation of the furosemide sensitive Rb^+-influx by bombesin is elevated in Ha-ras expressing cells. This is in accordance with the increased mitogenic activity of bombesin in Ha-ras expressing cells.

B 24

REGULATION OF THE PRODUCTION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS IN LUNG CANCER CELL LINES

Jaques, G., Wegmann, B., Havemann, K.
ZIM, Abt. Hämatologie/Onkologie der Universität Marburg

The inslulin-like growth factors (IGFs) are shown to be a group of growth factors in lung cancer, which fulfill all criteria of the autocrine/paracrine model. We and others have shown that besides the IGFs and their receptors for IGF-I and IGF-II, at least three different IGF-binding proteins (IGF-BPs) are produced by small cell lung cancer cell lines (SCLC) and non-small cell lung cancer cell lines (NSCLC). These IGF-BPs can enhance or inhibit the growth stimulating action of the IGFs. Here we investigated the influence of various hormones (polypeptide hormones, steroid hormones) and growth factors on the biosynthesis of these IGF-BPs by western ligand blotting and Northern blot analysis. Under serumfree non-stimulating conditions different protein expression patterns were found in SCLC and NSCLC cell lines. These patterns were changed by the incubation with androgens and IGFs in these cell lines. These findings lead to the hypothesis that the IGF-activity at the cellular level can be regulated by hormones or growth factors which influence the biosynthesis of the IGF-BPs.

B 25

THE FUNCTION OF DNA TOPOISOMERASES AND DNA POLYMERASES IN THE REPAIR OF UV-INDUCED DNA DAMAGE IN HUMAN FIBROBLASTS

Odilia Popanda and Heinz Walter Thielmann
Institute of Biochemistry, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, Federal Republic of Germany

In order to investigate which role **DNA topoisomerases I and II** play in the repair of UV-irradiated genomic DNA, we determined (i) endonucleolytic cleavage (using the alkaline elution technique) in normal fibroblasts and (ii) DNA repair synthesis (monitoring [α-^{32}P]dTMP incorporation) in saponin-permeabilized fibroblasts in the presence of specific inhibitors of DNA topoisomerases. To dissect the contributions of **DNA polymerases** α, β, δ, and ε, reduction of UV-induced DNA repair synthesis was measured under the influence of specific inhibitors of DNA polymerases. Dose-response experiments were performed throughout.

The inhibitor of **DNA topoisomerase I**, camptothecin, did not affect reparative DNA synthesis. However, several inhibitors of **topoisomerase II** such as novobiocin, coumermycin, *m*-AMSA, and quinacrine reduced [α-^{32}P]dTMP incorporation by up to 80 %. By contrast, other topoisomerase II inhibitors such as nalidixic acid, oxolinic acid, and etoposide did not exert effects. A similar inhibition pattern was found when DNA-incising capacity was monitored. It cannot be excluded that the first group of inhibitors indead interacted with topoisomerase II but also impeded later steps of the excision repair cascade. The second group of inhibitors, however, might have inactivated topoisomerase II, but this effect could have been masked by topoisomerase I which substituted for topoisomerase II. Therefore, combinations of inhibitors effective for both enzymes were used; camptothecin plus one of the following: nalidixic acid, oxolinic acid or etoposide. With these combinations, reduction of repair synthesis by 30 % was observed. These results suggest that topoisomerases (I or II) are involved in DNA repair steps preceding incision, but the action of the enzymes is not rate-limiting.

DNA repair synthesis was reduced by aphidicolin, butylphenyl-dGTP, butylanilino-dATP, and ddTTP. Comparison of inhibition constants with *in vitro* K_i values revealed that in permeabilized human fibroblasts reparative DNA synthesis is catalyzed by **DNA polymerase** ε.

B 26

HUMAN CASEIN KINASE II: THE STRUCTURE OF THE GENE ENCODING SUBUNIT BETA.

H. Voss[2], U. Wirkner[1], R. Jakobi[1], W. Ansorge[2] and W. Pyerin[1]
[1]Institute of Experimental Pathology, DKFZ, and [2]European Molecular Biology Laboratory, D-6900 Heidelberg, F.R.G.

The human casein kinase II (CKII) is an ubiquitous serine/threonine protein kinase with numerous functions in cell metabolism and growth. Special attention is being paid to CK II's ability to phosphorylate and regulate oncogene products such as Myc, Myb, Fos, adenovirus E1a, SV40 large T antigen, HPV E7 as well as tumor suppressor protein p53 and transcription factor SRF. CKII has a tetrameric structure; two catalytic subunits (α and α') form the holoenzyme together with two presumably regulatory subunits (β). We have isolated the gene encoding CKII subunit β from a human genomic DNA library and analyzed for its primary structure using exclusively non-radioactive procedures. The gene was found to span 4.2 kb and to be composed of seven exons with all the exon-intron junctional sequences conforming to the canonical GT-AG rule. Primer extension analysis determined three transcription initiation sites. The translation start is located early in the second exon; exon 1 is untranslated. The 3'-cleavage/polyadenylation signal sequence is in the last exon at position 4173 bp relative to the first transcription initiation site. The coding sequence for CKIIβ comprises 648 nucleotides identical in sequence to the published CKIIβ-cDNA sequence (Jakobi, Voss, Pyerin (1989) Eur. J. Biochem. **183**, 227-233). The upstream promoter region of the CKIIβ gene contains multiple potential gene regulatory sequence elements but lacks a TATA-box and a CCAAT box at standard position, which, together with other features, render the CKIIβ subunit gene a housekeeping gene. The CKIIβ gene promotor structure shares common features with that of other mammalian protein kinases.

B 27

ENHANCED EXPRESSION OF MITOCHONDRIAL AND CELLULAR GENES IN XERODERMA PIGMENTOSUM FIBROBLAST STRAINS FROM VARIOUS COMPLEMENTATION GROUPS

Heinz Walter Thielmann*, Manfred Rothe, and Dieter Werner#
*Institute of Biochemistry and #Institute of Cell and Tumor Biology, German Cancer Research Center, D-6900 Heidelberg, Federal Republic of Germany, Im Neuenheimer Feld 280

cDNA libraries were constructed from RNA of normal and xeroderma pigmentosum (XP) fibroblast strains (complementation groups A, C, D, and XP variant) in both the λ gt10 vector and in the *in vitro* transcription vector pBluescript. Approximately 16 000 plaques of each XP cDNA library in the λ gt10 vector were differentially screened with *in vitro* transcripts of cDNA in the pBluescript vector from both the same XP strain (under investigation) and the normal fibroblast strain. Ten differential clones were detected, and verified in a second hybridization round, to cause stronger signals with transcripts from XP strains than with those from the normal strain. The cDNA clones were analyzed on the sequence level; 7 of them coded for 4 mitochondrial genes: subunit I of cytochrome c oxidase (subunit of complex IV), apocytochrome b (subunit of complex III), and 16 S rRNA. One clone coded for a nuclear gene: the carcinoembryonic antigen. Two clones representing i) subunit I of cytochrome c oxidase and ii) 16 S rRNA diverged from the sequence of the human mitochondrial genome as present in the data base libraries. Clone i) exhibited a transition mutation (T > C) in position 7080 of the human mitochondrial DNA sequence corresponding to the exchange of phenylalanine by leucine. Clone ii) reflected a transcript of a mitochondrial genome rearranged in the 16 S rRNA gene.

The enhanced expression of mitochondrial genes in XP cells, together with the changes in DNA sequence, indicates that functions of the ATP-generating system were impaired. Whether this defect existed *ab initio* in XP individuals or originated from cumulative mutations due to lack of repair, cannot be decided. Nevertheless, human encephalomyopathies are known which result from mutant mitochondrial DNA. These disorders are characterized by several neurologic symptoms (among others) which are also common to XP: reduced intelligence, sensorineural deafness, ataxia etc. Thus, abnormal functions of mitochondria might be one explanation for the neurologic symptoms of XP.

B 28

Expression of the *Drosophila lethal (2) giant larvae* Tumor Suppressor Gene Product p127 in Insect Cells Using the Baculovirus System

D. Strand, A. Kalmes, H-P Walther, R. Ollo *,B. Mechler
Inst. f. Biochemie, DKFZ, D-6900 Heidelberg
*Inst. Pasteur, F-75724 Paris

We have used the baculovirus expression system to investigate the biochemical properties and biological function of the *lethal (2) giant larvae (l(2)gl)* gene product. Upon integration of the cloned *l(2)gl* cDNA sequence downstream of the viral polyhedrin promoter, full length *l(2)gl* protein of apparent molecular weight 127,000 was expressed. Here we report this protein is targeted to the membranes of infected cells, is found associated with the inner surface of the plasma membrane, and is immunologically indistinguishable from the p127 of *Drosophila* cells. Following cell disruption and partial membrane purification, this protein is retained in the membrane fraction after treatment with 1M NaCl, pH 11.5 and 6M Urea suggesting that the membrane attachment is by a similar mechanism as in *Drosophila* cells. These results indicate that the p127 protein expressed in baculovirus infected cells is biologically active and the availability of a large quanitity of presumably active *l(2)gl* gene product is a significant advance to understand the function of *l(2)gl* in tumor suppression.

B 29

CLONING OF A NOVEL CATALYTIC SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE

S. Wiemann, V. Kinzel and W. Pyerin
Institute for Experimental Pathology, DKFZ Heidelberg

The catalytic (C) subunit is the phosphorylating component of the cAMP-dependent protein kinase, a key element in a multitude of hormonally controlled cellular functions. The C-subunit, is known to be a group of isoforms comprising as yet Cα, Cβ, and Cγ. We isolated a full-length cDNA clone coding for a hitherto undiscovered isoform of the bovine C-subunit. The 5' end of the coding region and the 5' non-coding region of this 3365 bp clone are unique whereas the rest of the coding region and the 3' non-coding region are identical with isoform Cβ. The clone has therefore been named Cβ2. The deduced amino acid sequence of Cβ2 has a length of 397 amino acid residues and a calculated molecular mass of 46.1 kDa thus being some 6 kDa higher than that of any known C-subunit. The unique aminoterminal sequence of Cβ2 lacks the usual myristoylation site of C-subunits. It contains a stretch of hydrophobic residues and a stretch which may fold into an amphiphilic α-helix conceivably serving targeting functions. The existence of isoform Cβ2 is confirmed (i) by the isolation of a second independent Cβ2 clone, (ii) by the development of products of expected size and sequence upon amplification from total RNA of various bovine tissues with the polymerase chain reaction (PCR) using Cβ2-specific primers, and (iii) by Northern blots probed with a cDNA fragment containing exclusively Cβ2 sequence. Cβ2-mRNA has a size of 4.4 kb and is expressed in various bovine tissues, mainly in heart and brain. Both the size and tissue distribution are indistinguishable from that of Cβ-mRNA, thus explaining the failure of previous investigation to distinguish it from Cβ2. The deviation site is equivalent to the exon 1/exon 2 splice site of the mouse C-subunit. Since splice sites are highly conserved and since not a single mutation is found downstram of the deviation site, it is tempting to suppose that Cβ2 and Cβ are coded by one gene which possesses two alternatively spliced exons 1.

I 1

A PEPTIDE VACCINE AGAINST SIMIAN VIRUS 40 TUMORS.

R. Hess, H.-G. Hanagarth, C. Hässler, G. Brandner
Abt. Virologie, Institut für Medizinische Mikrobiologie & Hygiene der Universität, Freiburg, Postfach 820, D-7800 Freiburg (Germany)

The cell-membrane-located fraction of simian virus 40 (SV40) large tumor (T) antigen can act as an inducer as well as a target of cellular cytotoxicity against SV40-transformed cells. In place of the complete T protein, the synthetic carboxyterminal undecapeptide lys(698)-thr(708) ["KT"] was found to adequately exert both these functions [J. Cancer Res. Clin. Oncol. 115: S 44 (1989)]. In addition, KT is a B cell epitope recognized by a monoclonal antibody, PAb 1605 [EMBO J. 3: 1485-1492 (1984); Virology 166: 245-247 (1988)].

We show that KT can serve as a vaccine protecting mice against SV40 tumors. For this purpose KT is covalently ligated onto the cell membrane of living syngeneic (Balb/c) mouse spleen cells. Ligation is performed by 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP) generating the (ϵ-NH_2)PDP amide of lys (698) which is assumed to ligate KT with SH-groups of the cell surface. The linkage is confirmed by surface immunofluorescence or by PAb1605-mediated adherence of Dynabeads. When immunized intraperitoneally with these KT-decorated "pseudo-tumor" cells, mice become immune against a subcutaneous challenge with a tumorigenic dose of genuine SV40-transformed tumor cells (VLM), just like mice vaccinated with 60-Co-irradiated VLM cells. Tumor immunity was not obtained by injection of lymphocytes preincubated with free KT, nor by free KT plus incomplete Freund adjuvant nor by KT ligated to bovine serum albumin. Spleen cells from mice vaccinated with genuine or "pseudo"-tumor cells were cytotoxic in vitro both for genuine and "pseudo"-tumor cells.

Thus, covalent ligation with the cell membrane can convert a short synthetic epitope into a tumor-protecting vaccine.

(Supported by DFG [Br 281/11.2], BMFT [01GA8709] and Stiftung Müller-Fahnenberg)

I 2

AUGMENTATION OF TUMOR IMMUNOGENICITY BY NEWCASTLE DISEASE VIRUS DOES NOT REQUIRE VIRUS INFECTIVITY

Christian Ertel and Volker Schirrmacher
Institut für Immunologie und Genetik, DKFZ, 6900 Heidelberg

Newcastle disease virus (NDV) has been used succesfully to infect tumor cells to increase their immunogenicity. Clinical and animal studies with NDV-modified tumor vaccines applied postoperatively revealed antimetastatic immunotherapeutic effects. Furthermore, we have shown with metastatic mouse ESb lymphoma cells that NDV infected ESb cells elicit a better *in vitro* cytotoxic T cell (CTL) response than noninfected tumor cells. In ESb cells the investigated virus strain (NDV-Ulster) goes through only one complete replication cycle: adhesion to the host cell (ESb), fusion of virus with the host cell membrane, transcription, translation and replication of viral RNA, assembly of viral proteins at the host cell membrane and budding of the newly synthesised virions. The aim of these studies was to find out which of these events is essential for the observed augmentation of anti-ESb immunity? Our approach was to treat ESb cells with noninfectious NDV preparations, that lacked infectivity for different reasons: a) UV irradiation (NDV-UV), which destroys the viral genome and thus blocks the replication cycle at the level of RNA transcription/translation. b) NDV-F_0, harvested from the culture supernatant of NDV-infected ESb cells. In contrast to egg-grown NDV, such virions lack a functional fusion protein (F). The F-protein requires a certain processing event for functionality that does not occur in mouse cells as the host. Thus, NDV-F_0 is only capable to attach to the host cell. Its replication is blocked earlier than NDV-UV, at the stage of virus fusion with the host cell membrane. We found that both, NDV-UV and NDV-F_0, upon adsorption to ESb stimulator cells, are capable to augment the *in vitro* anti-ESb CTL response. The required dose for this "NDV-effect" was the same as with infectious NDV preparations. We conclude, that even membrane-attached virions (NDV-F_0) improve the recognition of the tumor cells by CTL. NDV could either improve lymphocyte-tumor cell contact via the viral adhesion molecule hemagglutinin (HN) or by altering the tumorcell surface via its neuraminidase activity, or both. It could also lead to the production of interferon(s) by responding cells in the culture.

I 3

ANTI-CD2 ANTIBODIES PREVENT CELL-MEDIATED IMMUNE RESPONSES IN VIVO

B. Gückel, V. Schirrmacher and B. A. Kyewski
Institute for Immunology and Genetics, German Cancer Research Center, D-6900 Heidelberg

The CD2 receptor functions as an adhesion and signal molecule in T cell recognition. Multimeric binding of CD2 on T cells to its physiologic ligant LFA-3 on cognate partner cells in vitro efficiently augments the antigen-specific T cell signal delivered by the T cell receptor/CD3 complex.

We analysed the role of CD2 in the murine immune response using a non-depleting anti-CD2 antibody which induces a marked, reversible modulation of CD2 expression on murine T and B cells *in situ*. This modulation is dose- and time-dependent, specific for CD2 and does not require the Fc-portion of the antibody. Anti-CD2 antibodies (rat IgG_1 or $F(ab')_2$) significantly suppress the CD4 T cell-mediated response to hen egg lysozcyme and the CD8 T cell effector response to a syngeneic tumor cell line. In both cases anti-CD2 antibodies are only effective, when administered before or within 24 h after antigen priming. The reduction of the anti-tumor response results in a 5-10 fold reduction of specific CTL precursor cells and the abrogation of protective anti-tumor immunity.

Beyond the effects of anti-CD2 antibodies on antigen-specific immune responses, a single antibody injection results in susstained unresponsiveness ("anergy") of T cells irrespective of antigen priming and CD2 modulation.

These results document (1) that CD2 participates in the early afferent phase of T cell-dependent immune responses *in vivo* and (2) that CD2-mediated signals influence the state of T cell responsiveness *in vivo*.

I 4

Immunosuppressive effects of TGF-ß in malignant gliomas may be reversed by TGF-ß-Phosphorothioate-antisense oligonucleotides in vitro.

Bogdahn, U, Jachimczak P, Schneider J, Brysch W , Schlingensiepen W ,Drenkard D, Behl C, Winkler J, Apfel R, Meixensberger J[6], - Dptms of Neurology and Neurosurgery[6], Univ. of Würzburg, MPI Biophysics, Univ. Göttingen[$], FRG

TGF-$ß_2$ is suppressing anti-tumor cytotoxic T-lymphocyte activation within malignant CNS-tumors *in vivo* and cytotoxic attack of $CD8^+$ -lymphocytes resp. proliferation upon stimulation with autologous tumor targets *in vitro*. In 2 glioblastoma early passage cell cultures (HTZ-153, HTZ-209) autologous cytotoxic -lymphocytes were enriched by in vitro mixed lymphocyte tumor cultures and expanded with IL-2. A polyclonal neutralizing anti-TGF-ß antibody did not enhance lymphocyte proliferation upon stimulation with lethally irradiated tumor targets (^{3}H-thymidine incorporation) and slightly stimulated lymphocyte cytotoxicity against autologous targets (as determined by a colorimetric assay). Preincubation of target cells for 24 hrs with TGF-ß-phosphorothioate-antisense-oligonucleotides (derived from a TGF-$ß_2$- sequence, f.c. 1uM, 14-mer) however enhanced lymphocyte proliferation (up to 3-fold) and autologous tumor cytotoxicity (preliminary experiments, up to 50%). These observations may have implications for *in vitro* - expansion and *in vivo* activation of specific autologous $CD8^+$ - lymphocytes within malignant gliomas.

I 5

LOCAL CELLULAR IMMUN REACTIVITY PATTERNS IN GASTRIC CANCER PATIENTS, A COMPARETETIVE STUDY

K. Stulle, P. Marquardt, H. P. Vollmers, J. Müller and H.-K. Müller-Hermelink.
Pathologisches Institut der Universität Würzburg

The reaction of peripheral blood (PBL) and lymphocytes out of regional metastatic (RLNL+) or non metastatic (RLNL-) lymph node and tumor infiltrating lymphocytes (TIL) were evaluated in gastric cancer. We determined the precursor frequency and the reactivity of T-cells to autologous tumor cells. Peripheral blood lymphocytes showed the highest natural killer (NK) activity thus indicating the presence of many NK cells in this compartment. The precursor frequency was low (only 3%) and whole blood stimulation tests revealed that PBL of cancer patients had a low ability to proliferate even after stimulation with mitogens. RLNL- showed the highest proliferative frequency of all four groups. As compared to PBL, RLNL+ had equal proliferation capacity. The precursor frequency of NK cells and their capacity to lyse NK sensitive target cells (K562) in RLNL+ and RLNL- were lower then in PBL. The proliferation frequency of tumor infiltrating T-lymphocytes (TIL) was relatively low to normal PBL and a lack of NK activity was detected.
With our cloning conditions [low dose interleukin 2 (100 IU/ml)] no specifically killing T-cells can be found whithin all four groups.
By using autologous Mixed Lymphocytes-Tumor-Culture (MLTC) in TIL and PBL no specific cytotoxic T-cells were detected. Only in lymph node cell population using the same conditions the proliferation of specific cytotoxic T-cells can be observed.

I 6

Construction and expression of an antibody/enzyme fusion molecule for antibody dependent enzyme prodrug therapy (ADEPT).

P. Lorenz[+*], M. Schuermann[+] and G. Seemann[*]
* Behring Research Laboratories, 3550 Marburg
+ Institute for Molecular Biology and Tumor Research, 3550 Marburg, FRG

ADEPT has been developed with the aim to target the toxic effects of chemotherapy to the tumor site. To achieve this principle monoclonal antibodies (MAbs) are used as vehicles to deliver enzymes specifically to the tumor. The specific approach of our group was to link human ß-glucuronidase (Oshima, A. et al. 1987) to the humanized form of the murine MAb BW 431/26 (Seemann et al. in preparation) using recombinant DNA technology (Bosslet et al. 1991, this issue). The murine MAb BW 431/26 is highly specific for membrane associated CEA and is successfully applied in immunoscintigraphy for detection of CEA-positive tumors.
A part of the immunoglobulin heavy chain gene was fused to a modified cDNA coding for the mature form of human ß-glucuronidase. The construct was cloned in an expression vector for eucaryotic cells and transfected in BHK-cells together with the humanized BW 431/26 light chain gene in BHK-cells using calcium phosphate precipitation. Transfectoma supernatants were tested for the presence of immunoglobulin fragments and transfectoma clones were identified which secrete the MAk/enzyme fusion molecules (Bosslet et al. 1991, this issue).

The strategy for the construction of the MAk/enzyme hybrid gene will be presented.

Oshima et al., Proc. Natl. Acad. Sci. USA, (1987) 84:685-689

I 7

MONOCLONAL ANTIBODY UM-1H12 SHOWS DIFFERENT IMMUNOBINDING TO NUCLEI OF NORMAL AND MALIGNANT CELLS

[1]Angelika Ptok, [1]M. Ptok, [2]T.E. Carey
[1]Univ.-HNO-Klinik Tübingen; [2]Kresge Hearing Research Institute, Univ. of Michigan, Ann Arbor, MI 48109, USA

In the course of raising tumor specific monoclonal antibodies to the VX2 squamous cell carcinoma of rabbits, several monoclonal antibodies were produced. Hybridoma UM-1H12 was isolated after immunization with live VX2 and ELISA screening on cultured VX2 and rabbit fibroblasts as target cells. In ELISA the antibody showed a strong binding to the carcinoma cell-line VX2 but also to a lesser degree to fibroblasts. In immunocytochemical assays with UM-1H12 on fixed rabbit cell cultures (UM-VX2, rabbit fibroblasts) or frozen tissue sections (rabbit VX2 carcinoma, normal tissue) a specific staining of nuclei of both normal and malignant cells could be observed. When tested against guinea pig and human cells (guinea pig fibroblasts, human squamous carcinoma cells, guinea pig skin, muscle, gut etc) the same degree of intense nuclear labeling was obtained. In mitotic nuclei the antibody stained condensed chromosomes but not other parts of the nucleus and in interphase the staining was diffusely distributed throughout the nucleus. In normal cells the nuclear staining was uniform in all nuclei but in the VX2 carcinoma and in human squamous carcinomas of the head and neck the staining varied greatly between cells.
The different pattern of nuclear staining in the various phases of mitosis indicates that the UM-1H12 epitope is a chromatin-associated antigen. The variation in staining between cells in neoplastic lesions compared to the uniformity in normal cells indicates that the antigen exhibits an abnormal expression pattern in malignant cells. In addition to DNA and histone proteins, possible nuclear antigens include nuclear protooncogene products such as c-myc, c-fos and c-jun which are expressed only transiently during the cell cycle (although the uniform staining of interphase nuclei in normal tissues tends to rule out cell cycle phase specific antigens), other DNA binding proteins that regulate gene expression such as the p105 retinoblastoma tumor suppressor gene product, or the Wilms' tumor suppressor gene product. It is likely that the UM-1H12 antigen is important in normal nuclear functioning because it is conserved phylogenetically and because its expression is altered, relative to normal cell types, in both human and rabbit squamous cell carcinomas.

I 8

TNF-ALPHA GENE EXPRESSION IN CELLS OF SMALL CELL LUNG CARCINOMA LINES

Steffen, M., Nitz, U.C., Everding, B., and Hölzel, F., Dept. of Internal Medicine and Dept. of Phys. Chemistry, University Hospital Eppendorf, Hamburg

Tumor Necrosis Factor alpha (TNF) is a multifunctional cytokine with a broad range of effects. It can act as a mediator in the regulation of inflammatory processes, of the immune response and of tumor cell growth. The expression of the TNF gene was analysed in 6 small cell lung cell carcinoma lines. Northern blot analysis revealed constitutive TNF mRNA production in 2 of the lines. Only one of these lines produced TNF protein which was biologically active as shown in a bioassay using the TNF sensitive cell line WEHI 164. One of the cell lines without constitutive TNF mRNA expression could be induced to TNF mRNA production by cycloheximide.
The results show that there are cells of small cell lung carcinoma lines which produce TNF mRNA and the biologically active cytokine. In other cells of this differentiation only TNF mRNA is detectable, and obviously translation of the mRNA or secretion of TNF protein is blocked. Furthermore, there are cells of small cell lung carcinomas without TNF gene transcription. Obviously the TNF gene expression is differentially regulated in this cell type. The meaning of the TNF production by these tumor cells is not understood. Possibly, it plays a role in the regulation of tumor cell growth or differentiation.

I 9

IMPACT OF TNF-α ON THE MIGRATION BEHAVIOUR OF LYMPHOCYTES

G.Kantwerk-Funke, G.Boll, K.S.Zänker, Inst.f.Immunologie, Univ. Witten/Herdecke, Stockumerstr.10, 5810 Witten, FRG

Beside the well known cytotoxic effect of TNF-α on tumour cells in vitro there are some indications that this cytokine modulates the effector function of immune cells. We are focusing our interest on the migration behaviour of immune competent cells influenced by endocrine, neuroendocrine and paracrine reacting substances. Therefore we investigated the properties of TNF-α in respect to lymphocyte migration. We studied the two-dimensional (2D) migration in a thin glass chamber within an established heat gradient and the three-dimensional (3D) migration into a collagen gel. After incubation in medium with TNF-α (50 ng/ml, 4-5 days) the mean velocity of human peripheral blood lymphocytes (PBL) was highly increased in a 2D-approach. Native PBL exhibited a mean velocity of 5.4 µm/min and a positive thermotaxis. TNF-α treated PBL increased significantly their mean migration speed to 7.6 µm/min but did not demonstrate a thermodependent orientation. In order to exclude the possibility of failure by undefinable prestimulation of PBL in vivo we used spleen lymphocytes of SPF-Balb/c mice for the 3D-experiments. TNF-α incubated spleen lymphocytes on top of the gel revealed a deeper immigration into the gel compared to native spleen cells within the first 12 hours. Extending the incubation time to 72 hours we observed an immigration depth gradually decreasing to about 60% of the untreated control (100%). Using the same experimental design, untreated lymphocytes migrated within the first 24 hours into collagen gels, unrespectively of a TNF-α presence in the gel. Extending the migration time the immigration depth from top of the layer into the TNF-α containing gel was again reduced (60%) compared to TNF-α-free gel (100%). No dose dependence could be observed (1-100 ng TNF-α/ml). Taking together the results of the 2D and 3D experiments suggest, that TNF-α on one hand enhances the migration velocity of lymphocytes, but on the other hand TNF-α exhibits a trapping-like effect on migrating lymphocytes. This obviously contradictory behaviour of lymphocytes in the presence of TNF-α becomes plausible in regard to the paracrine role of this cytokine. Therefore TNF-α increases the probability of antigen contact of tissue infiltrating lymphocytes by accelerating the motility; concomitantly inhibiting the evasion of lymphocytes out of the affected tissue but by trapping them locally.

I 10

MACROPHAGES IN TUMORS OF BRAIN ORIGIN DO NOT EXPRESS INTERLEUKIN-1 OR TUMOR NECROSIS FACTOR-ALPHA.
Immunohistochemical Investigation

Hahn P¹, Kist A², Kirsch M¹, Fischer H², Buttler A² and Schachert G¹
¹Neurochirurg.Univ.-Klinik Heidelberg,²Inst.Immunol.DKFZ,³Inst.Virol.DKFZ

The brain is an immunologically privileged site. However, we know that in brain tumors are numerous macrophages and lymphocytes. The goal of our experiments was to visualize the cellular immune response in different brain tumors and to determine the state of macrophage activation by Interleukin-1 (Il-1)and tumor necrosis factor-alpha (TNF) expression.

The ABC-peroxidase method was used for immunohistochemistry. Four different tumor groups were investigated : meningiomas, glioblastomas, low grade gliomas, and brain metastasis. For staining of astrocytes, macrophages, lymphocytes, interleukin-1- and tumor necrosis factor-expression different monoclonal and polyclonal antibodies were tested. As control for Il-1- and TNF- expression activated and non-activated peripheral blood monocytes were used. Serial frozen sections of 5µm thickness were performed.

Results: We saw infiltrates of lymphocytes and macrophages in glioblastomas, metastases , and meningiomas while in low grade astrocytomas were only a few. Il-1- or TNF- expression was investigated as sign for macrophage activation. Only in metastases we detected some cells staining for Il-1 which could be correlated to macrophages. In tumors of brain origin we did not find any staining of Il-1 or TNF associated to macrophages.

I 11

Oncogene Expression in Human Lung Tumor Cell Lines of Various Differentiation

B.Everding*, M.Steffen*, F.Hölzel**
Departments of Internal Medicine*, Physiological Chemistry and Obstetrics and Gynecology**, University Hospital Eppendorf, Hamburg, FRG

Cell lines derived from large cell carcinoma (SK-LC-3), squamous cell carcinoma (SK-LC-14), adenocarcinoma (SK-LC-15) and small cell carcinoma (NCI-H69) of the lung have very different patterns and levels of expression of cellular oncogenes. While K-ras-specific mRNA was expressed in proliferating cells of all cell lines examined, c-myc mRNA was found in SK-LC-3, SK-LC-14 and SK-LC-15 cells. C-fos expression was determined in SK-LC-3- and SK-LC-15 cells. C-fms mRNA coding for CSF-1 receptor characteristic for hematopoietic cells, was expressed only in SK-LC-14 squamous carcinoma cells.
In kinetics experiments, the proliferation of SK-LC-14 cells was inhibited by IFN γ (5000 U/ml). At the onset of the antiproliferative effect of IFN γ, an increase of c-myc and c-fms mRNA was demonstrated unexpectedly. Thus the level of c-myc mRNA expression, largely considered as indicator of cell proliferation, appeared to be negatively correlated with SK-LC-14 cell growth. The exposure of SK-LC-15 adenocarcinoma cells to IFN γ led to inhibition of cell proliferation and down-regulation of c-fos expression. Apparently, lung tumor cells of various differentiation may show various expression patterns in response to treatment with IFN γ.

I 12

Expression of the CD56 (NKH-1) and the CD57 (HNK-1) Antigen on Lung Cancer Cell Lines

J. Heymanns, C. Hennig, M. Rotsch, K. Havemann. Dept. Internal Medicine, Div. Hematology/Oncology, Baldingerstrasse, D-3550 Marburg, Germany

Small cell lung cancer cell lines (SCLC) express a number of neuroendocrine features, which non small cell lung cancer cell lines (NSCLC) generally lack. The neural cell adhesion molecules (NCAM, three isoforms with 180, 140, 120 kDa) were first found in nervous tissues and are present in neuroendocrine tumors too. The presence of (NCAM) has been previously demonstrated in SCLC (Aletsee-Ufrecht et al. 1990, FEBS 267, 295). Lanier et al. (1989, J. Exp. Med. 169, 2233) showed, that the 140 kDa isoform of NCAM is identical to the CD56 antigen, which is present on NK-cells. SCLC and NSCLC cell lines were analysed by flow cytometry and immunoblot for the expression of CD56 and CD57, which is a carbohydrate epitope on the NCAM antigen. All SCLC cell lines but one with adhesive growth properties were stained by CD56, the coexpression of CD57 varied considerably with two SCLC-V being negative. CD56 detected a 120-125 kDa and a 180-185 kDa protein in the immunoblot, but not the previously described 140 kDa isoform of NCAM. CD57 detected a 120-125 kDa protein in the immunoblot probably the low molecular form of NCAM. NSCLC were CD56 negative with one exception. A LCLC cell line was found CD56 positive. The importance of NK-markers on tumors cells for the immunological defence is unkown and should concern further studies.

I 13

Jürgen R. Fischer, Sabine Stehr, Harald Lahm[1], Peter Drings and Peter H. Krammer[2]

Thoracic Clinic, Department of Medical Oncology, Amalienstraße 5, 6900 Heidelberg-Rohrbach, FRG, [1]ISREC, Ch. des Boveresses 155, 1066 Epalinges, CH and [2]German Cancer Research Center, Division of Immunogenetics, 6900 Heidelberg, FRG

Small cell lung cancer (SCLC) lines release soluble mediators with autocrine and immunosuppressive activity

To understand the biology of malignant growth the phenomena of autocrine growth stimulation have to be further elucidated. In addition, the interactions between tumor cells and the immune system on a molecular level have to be understood. Therefore, we investigated the effects of soluble mediators secreted by SCLC lines on growth of the producer cell and on modulation of cytokine-induced growth of lymphocytes. We found that proliferation of SCLC is induced by a mediator(s) in the conditioned medium of SCLC lines. This autocrine activity is different from bombesin and IGF-I. In addition, T cell growth is suppressed by a factor(s) in SCLC-conditioned medium. Initial biochemical characterization of the immunosuppressive activity shows that this factor is dialysable, partially sensitive to temperature, pH 2 and 10, and is partially destroyed by proteinases.

I 14

MONOCYTE KILLING OF MALIGNANT BRAIN TUMOR CELLS - FIRST RESULTS.

KIRSCH M[1], STRUBEL A[2], KIST A[2], HIHN R[2], FISCHER H[3], BUTTLER A[1] AND SCHACKERT G[1]
[1]Neurochirurg.Univ.-Klinik Heidelberg, [2]Inst.Radiol.u.Pathophys.DKFZ, [3]Inst.Immunol.DKFZ, [4]Inst.Virol.DKFZ Heidelberg

In vitro and in vivo experiments with activated human peripheral blood monocytes showed enhanced tumoricidal activity against extracerebral tumor cells whereas normal cells stay unharmed. Monocytes were activated with biological response modifiers (BRM).

Peripheral blood monocytes from healthy donors were isolated by density separation and adherence. Following an incubation period of 24h in endotoxin-free medium and in the presence of BRMs (gamma- and beta- interferon, tumor necrosis factor-alpha, muramyl dipeptide) activated monocytes were tested against ^{3}H-thymidine labelled target cells to provide a target:effector cell ratio of 1:20.

Activated monocytes were tested against 5 different malignant glioma cell lines.
The activation with lipopolysaccharide served as control (cytotoxicty rate about 63%).

We found high cytotoxicity rates after activation with beta-interferon (46% ±5) and especially tumor necrosis faktor-alpha (72% ±2). The combined effect of tumor necrosis factor-alpha plus beta-interferon decreased monocyte cytotoxicity compared to single agents. Combination of beta-interferon or tumor necrosis factor-alpha with gamma-interferon or muramyl dipeptide enhanced monocyte tumoricidal activity compared to single agents.

I 15

REGULATORY FUNCTION OF PROTEIN KINASE C IN THYMOCYTE APOPTOSIS

S. Friedenauer, G. Fürstenberger, D. Lindner and F. Marks
Institut für Biochemie, Deutsches Krebsforschungszentrum Heidelberg

Apoptosis in thymocytes can be induced by a variety of exogenous stimuli such as glucocorticoids, Ca ionophores, cAMP-elevating agents and phorbol esters .Thus, multiple mechanisms may be involved in this process. The apoptosis-inducing activity of phorbol esters indicates a critical role of protein kinase(s) C in apoptosis. On a molar base the phorbol ester TPA turned out to be as effective in inducing apoptosis as the glucocorticoid FA (ED_{50}=0.5 nM). The main difference between both agents was an 8 hr lag phase of the apoptotic effect observed upon TPA but not upon FA treatment. This lag phase coincided with the kinetics of PKC down modulation in TPA-treated thymocytes, as measured by Western blotting and assaying enzyme activity. Both apoptosis and PKC down modulation could be inhibited by cycloheximide.
Since TPA-dependent activation of PKC resulted in an inhibition of FA-induced apoptosis, it is concluded that thymocytes are protected from apoptosis by active PKC and that PKC down modulation is a prerequisite for phorbol ester-induced apoptosis. Whether the inactivation and down modulation of PKC observed also upon FA treatment is causally related to glucocorticoid-induced apoptosis remains to be shown.

K 1

STEREOCHEMICAL INFLUENCES IN THE REACTION OF CHIRAL EPOXIDES WITH NUCLEIC ACIDS INDICATING DIFFERENT GENOTOXIC POTENTIAL OF THE ENANTIOMERS

H. Peter, B. Marczynski, H. Karels, H.W. Goergens

Chiral epoxides are generated in the organism of man and animals from the most widely used plastic monomers propylene, vinyl chloride, acrylonitrile, 1,3-butadiene and styrene. These reactive epoxides are suspected to initiate the transformation process in somatic cells to tumour cells by binding to DNA. In the discussion of mechanisms of genotoxicity, the influence of stereochemical differences in the interaction of chiral (in terms of configuration) epoxides with chiral (in terms of conformation) DNA has hitherto not been taken into consideration. In contrast, numerous examples of enantioselective reactivity between chiral molecules have been described in pharmacology and biochemistry of natural compounds.

Both enantiomers of styrene 7,8-oxide and propylene oxide were incubated with synthetic homobasic polynucleotides, oligonucleotides and calf thymus DNA. With the methods of equilibrium dialysis, determination of the melting temperature of double stranded nucleic acids, circular dichroism spectroscopy and hplc separation of specific alkylation products with nucleosides, such enantioselective reactions were investigated. Homobasic single stranded polynucleotides were preincubated with the pure enantiomers of both epoxides and subsequently hybridized with their complementary or noncomplementary unreacted oligonucleotides in the equilibrium dialysis experiments. Both enantiomers of each epoxide investigated influenced hydrogen bonding in the recombined double strands differently. Although both enantiomers of propylene oxide and styrene oxide enhanced the melting temperature of preincubated calf thymus DNA, this shift was significantly higher for each R-enantiomer in comparison to its corresponding S-enantiomer. Enantioselective reactivity was also confirmed through investigation of pretreated homobasic polynucleotides with circular dichroism spectroscopy, either in the single stranded form or after hybridization to the double strands. Finally, when aliquots of the preincubated polynucleotides were hydrolyzed enzymatically with subsequent hplc separation of the nucleosides, an enantiospecific binding with uridine was observed in the case of S-styrene oxide and with cytidine in the case of R-propylene oxide.

From the results presented it can be concluded that enantiomers of chiral epoxides show a different reactivity towards nucleic acids. The determination of enantioselective alkylation products with constituents of nucleic acids indicates a difference in the genotoxic potential of the enantiomers. This stereochemical phenomenon may also play a role in the interaction of the intermediate chiral epoxides from vinyl chloride, acrylonitrile and 1,3-butadiene. The combination of equilibrium dialysis, melting behaviour and circular dichroism spectroscopy with hplc analysis provides an in vitro test system well suited for the investigation of such chiral effects in genotoxicity.

Institut für Arbeitsphysiologie, Ardeystr. 67, 4600 Dortmund 1, FRG

K 2

DNA BASE MODIFICATIONS INDUCED BY SINGLET OXYGEN ARE RECOGNIZED BY FPG PROTEIN

B. Epe, E. Müller, D. Schütze and [1]S. Boiteux
Institute of Toxicology, University of Würzburg, FRG and [1]Institute Gustave Roussy, 94805 Villejuif, France

Hydroxyl radicals and singlet oxygen represent two different types of reactive oxygen species that can be generated in and by biological systems and are known to react with DNA. In contrast to the situation for hydroxyl radicals, little is known about the chemical nature and the repair of singlet oxygen-induced DNA modifications.

We modified supercoiled DNA from bacteriophage PM2 in a cell-free system by exposure to (i) hydroxyl radicals, generated by xanthine and xanthine oxidase in the presence of Fe^{III}-EDTA and (ii) singlet oxygen, generated either by thermal decomposition of $NDPO_2$, an aromatic endoperoxide, or by methylene blue in the presence of light, a photosensitizing system. Subsequently, we determined the number of single strand breaks and of various repair endonuclease-sensitive sites. The results indicate that the damage induced by singlet oxygen consists predominantly of base modifications which are recognized by formamidopyrimidine-DNA glycosylase (FPG protein), a repair endonuclease so far known to recognize only imidazole ring-opened purines. Only very few sites of base loss (AP-sites), single strand breaks and sites sensitive to endonuclease III (dihydropyrimidine derivatives) are present in the singlet oxygen-modified DNA, in marked contrast to the DNA damaged by hydroxyl radicals. Using HPLC and an electrochemical detector, 8-hydroxyguanine was found to be excised from the singlet oxygen-modified DNA by FPG protein, indicating for the first time that this promutagenic lesion is subject to repair by a glycosylase.

K 3

Formation of deoxyguanosine adducts as underlying mechanism for mutagenicity and carcinogenicity of β-alkyl substituted acroleins

E. Eder, C. Deininger and C. Hoffman
Institut für Toxikologie, Universität Würzburg

The importance of acrolein and its congeners as environmental pollutants is well-known. In the last few years these substances have gained increased interest as genotoxic products of lipid peroxidation, lipid and arachidonic acid oxidation and of reactive oxygen species. Some of them were revealed to be carcinogenic. Unfortunately, contradictory results have been reported on the mutagenicity of these compounds. The difficulties in Salmonella mutagenicity testing depend on the high toxicity of these substances towards bacteria. When using a threefold bacterial density in the Ames preincubation test we found unambiguous mutagenicities for crotonaldehyde, pentenal, hexenal, heptenal and 2,4-hexadienal but no mutagenicity for the extremely toxic 2,6-nonadienal. We also investigated the formation of deoxyguanosine adducts of these compounds (except nonadienal) and could identify and characterize cyclic 1,N^2-deoxyguanosine adducts, cyclic N7, C8-guanine adducts, linear N7-guanine adducts and biscyclic 1,N^2, 7,8 adducts as well as cyclic, linear 1,N^2, N7 bisadducts. There was a good correlation between the formation of adducts and mutagenicity. Adduct formation is most probably the underlying primary mechanism for mutagenicity and carcinogenicity.

K 4

FREQUENT ACTIVATION OF c-Ki-*Ras* BY POINT MUTATION IN PANCREAS CANCER OF SYRIAN HAMSTER BUT NOT IN ADENOMAS OR CARCINOMAS OF RAT PANCREAS

E. Scherer[1], E. Vermeulen[1], H.J. van Kranen[2], J. Bax[1], R.A. Woutersen[3] and C.F. van Kreijl[2]

[1] Division of Chemical Carcinogenesis, The Netherlands Cancer Institute (Antoni van Leeuwenhoek Huis), Amsterdam, [2] Laboratory of Carcinogenesis and Mutagenesis, National Institute of Public Health and Environmental Protection, Bilthoven. [3] Department of Biological Toxicology, TNO-Toxicology and Nutrition Institute, Zeist; The Netherlands.

We have investigated the activation of *ras* genes by point mutation at codons 12, 13 or 61 in experimentally induced pancreas tumors of rat and Syrian hamster. Mutations were detected by allel-specific oligomer hybridization of *ras* sequences amplified from tumor DNA by the polymerase chain reaction. In rat no mutations were observed in c-Ki-*ras* or c-Ha-*ras* of 9 adenomas and 15 carcinomas of acinar origin indicating that activation of these genes is not a frequent event in azaserine-induced rat pancreas carcinogenesis. In 19 out of 20 adenocarcinomas of hamster pancreas codon 12 (85%) or 13 (10%) of c-Ki-*ras* was mutated. All mutations were G->A transitions of the second base in the codons involved, leading to substitutions for glycine by aspartic acid. The consistency of this mutation pattern with DNA methylation-induced mutagenesis suggests that point mutation of c-Ki-*ras* is induced by the methylating carcinogen *N*-nitrosobis(2-oxopropyl)amine (BOP), and thus could represent the first event in hamster pancreatic carcinogenesis. Our results underline the relevance of the BOP-Syrian hamster system as experimental model for human pancreatic cancer.

K 5

CORRELATION BETWEEN CONVERSION AND 8-LIPOXYGENASE-MEDIATED CLASTOGENESIS IN TPA-TREATED MOUSE SKIN

B. Schurich, G. Fürstenberger, H.Hagedorn, E. Besemfelder and F. Marks
Institut für Biochemie, Deutsches Krebsforschungszentrum Heidelberg

Initiation-promotion provides a well-established experimental approach of two-stage carcinogenesis. In mouse skin, initiation is achieved by single treatment with a chemical carcinogen such as 7,12-dimethylbenz(a)anthracene (DMBA) while promotion is carried out by chronic application of irritant skin mitogens such as the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). Moreover, tumor induction in initiated skin can be subdivided in at least two stages brought about by subsequent treatment with TPA (converting tumor promoter; conversion stage) and mezerein (Sencar mice) or 12-O-retinoylphorbol-13-acetate (RPA; NMRI and CD-1 mice) so called non-converting tumor promoters (promotion stage). A striking difference between the non-converting promoter RPA and the converting promoter TPA is that only the latter induces chromosomal aberrations in mouse epidermal cells in culture. Based on this finding, chromosomal alterations or damage have been proposed to play a role in the conversion stage of skin carcinogenesis. This view is supported by the observation that the converting promoter TPA but not the non-converting promoter RPA induced chromosomal aberrations when applied to mouse skin in vivo , i.e. under the conditions of conversion. Here we present evidence that TPA-induced conversion and clastogenesis can be mediated by metabolites derived from 8-lipoxygenase-catalyzed arachidonic acid oxygenation. TPA has been shown to induce an epidermal 8-lipoxygenase activity. Moreover, the products of this enzyme reaction 8-hydroperoxy- as well as 8-hydroxyeicosatetraenoic acid have been found to induce chromosomal aberrations in mouse keratinocytes. Prevention of lipoxygenase-catalyzed arachidonic acid metabolism impairs both the clastogenic as well as the converting activity of TPA. These findings and the fact that the time point of maximum inhibiton of conversion by lipoxygenase inhibitors coincides with the maximum of TPA-induced 8-lipoxygenase activity point to a critical function of 8-lipoxygenase-derived arachidonic acid metabolites as endogenous mediators of phorbol ester-induced conversion.

K 6

LONG-CHAIN N-NITROSO-N-METHYLALKYLAMINES OCCURENCE AND ANALYSIS

E. Kamp, G. Eisenbrand
Food Chemistry and Environmental Toxicology
University of Kaiserslautern

Unsymmetrically substituted, long-chain N-nitroso-N-methylalkylamines are contaminants in cosmetics, household cleaning preparations and light duty dishwashing liquids [Hecht et al., 1982; Morrison & Hecht, 1983; Morrison et al., 1983]. Information on potential contamination of products from the German market is not available yet. An analytical procedure for screening of a broad spectrum of commercial consumer products was developed. Examination of 86 products from the German market did not indicate significant contamination. In contrast, analysis of a limited number of samples [26] from the US market revealed the presence of methylalkylnitrosamines in hair care and dishwashing preparations containing fatty amine oxides. Fatty amines and the corresponding N-oxides act as nitrosamine precursors. Investigations on factors of relevance for nitrosamine formation and it's prevention are in progress.

K 7

NITROSODIETHANOLAMINE IN CUTTING FLUIDS

Spiegelhalder B.[1] Bolm-Audorff U.[2] Bienfait H.G.[2] & Preussmann R.[1]
[1]Institute of Toxicology & Chemotherapy, German Cancer Research Center, Heidelberg, FRG. [2]Ministery of Social Welfare, Dept. of Occupational Safety, Division of Occupat.Medicine and Div. of Epidemiology. Wiesbaden, FRG.

Since more than 10 years it is known that cutting fluids can contain considerable amounts of carcinogenic N-nitrosodiethanolamine (NDElA). Recently investigated grinding and cutting fluids, however, still showed high NDElA levels. The introduction of legislative measures had not lead to a recognizable improvement. In order to get representative data about the present exposure situation to NDElA a survey was conducted in cooperation with the Governmental Hygienist in Hessen.

A total of 378 samples were taken in 91 factories of the metal working industry. About 1537 cutting, drilling, grinding and sawing workplaces were investigated.

Only 13 samples had no detectable NDElA (lower limit of detection was 0.1 ppm), 68 samples contained levels from 0.1 to 0.9 ppm NDElA, 148 were between 1 and 4.9 ppm NDElA. Levels above 5 ppm NDElA were found in 149 samples, of which 13 samples exceeded the limit of 500 ppm. The highest concentration was found in a cooling liquid concentrate with 2.2 percent NDElA.

Nitrite levels in most samples were below 100 ppm and originated probably from nitrate in water by bacterial reduction. Some samples, however, contained nitrite up to 29 percent as an anti-corrosive agent.

In a follow-up study also air measurements and biological monitoring were performed to investigate workers exposure. NDElA concentration in air was between 0 and 2.8 $\mu g/m^3$. In urine after an exposure free weekend no NDElA was detectable, whereas up to 15 ppb NDElA was found after exposure by cutting fluids. Air measurements and biological monitoring showed no correlation. It is assumed that exposure is mainly due to direct skin contact with cutting and grinding fluids.

K 8

SAFE AMINE DERIVED VULCANIZATION ACCELERATORS AS A MEANS TO REDUCE HIGH NITROSAMINE LEVELS IN THE RUBBER INDUSTRY

C.-D. Wacker, R. Preussmann, H. Kehl & B. Spiegelhalder
Institute of Toxicology & Chemotherapy, German Cancer Research Center, Heidelberg, FRG.

The occurrence of carcinogenic nitrosamines in the rubber and tyre industry is still an unsolved problem. Air measurements in German rubber factories throughout 1988-89 showed predominantly N-nitrosodimethylamine (NDMA), N-nitrosodibutylamine (NDBA) and N-nitrosomorpholine (NMOR) in concentrations up to 41 $\mu g/m^3$.

Exposure to these carcinogens is caused by the industrial application of certain vulcanization accelerators such as tetramethylthiuramdisulfide (TMTD) or zinc-dibutyldithiocarbamate (ZDBC). These amino derivatives are readily nitrosated by various nitrosating agents at elevated temperatures, i.e. under curing conditions. In a project started 3 years ago we looked for alternatives to these and other commercial accelerators. The substitutes should be good acting accelerators but unable to form carcinogenic by-products. Since then, about 140 novel vulcanization accelerators derived from safe amines have been synthesized. Safe amines are characterised in our understanding as forming only non-carcinogenic nitroso compounds if nitrosated. In addition, some safe amines are much less nitrosatable than 'normal' amines due to steric hindrance.

As a result, we have developed a number of safe amine derived accelerators such as bis-(4-methyl-piperazino)thiuramdisulfide ("Vulcasafe MPT") and zinc-(4-methyl-piperazino)dithiocarbamate ("Vulcasafe ZMP"), which only slightly differ in their vulcanization behaviour from the technical standards in their classes. In nitrosation studies under simulated vulcanization conditions no other nitroso compounds than the 'safe' nitrosamines corresponding to the amino components in the accelerator molecules could be found. Thus, with the new accelerators a substancial reduction of carcinogenic nitrosamine exposure in the rubber industry is now accessible.

This work was fully supported by the German Ministry of Research and Technology (Grant No. 01HK496/1).

K 9

N-NITROSAMINES IN AMBIENT AIR OF INDUSTRIAL AREAS IN GERMANY.

Z. Akkan, R. Preussmann & B. Spiegelhalder.
Institute of Toxicology & Chemotherapy, German Cancer Research Center, Heidelberg, FRG.

Studies of occupational and environmental exposure to carcinogenic N-nitroso compounds revealed high levels of airborne nitrosamines in working places of industrial users of amines, and considerable contaminations can still be found in the ambient air nearby. To have a reliable survey of the emission situation in the FRG, a measuring programme has been set up, which includes systematic screening for volatile nitrosamines in the ambient air adjacent to certain industries in the whole country.

From July 1990 to January 1991 a total of 310 air samples was collected in industrial areas mainly in the middle west and south west of the country. Plants of the following branches were investigated: waste recycling industry, chemical industry, metal working industry, founderies, rubber industry, paper industry, tanneries, fish processing industry, mining industry.

Nitrosamines were determined by gas chromatography in combination with chemoluminescence detection using validated analytical procedures. About 25% of the air samples were found to contain N-nitrosamines with levels between 0.01 and 0.09 $\mu g/m^3$ of N-nitrosodimethylamine, N-nitrosodiethylamine, N-nitrosodipropylamine, N-nitrosodibutylamine and N-nitrosomorpholine. In some cases the presence of nitrosamines typical for the investigated industrial group could be confirmed. The highest frequency of contaminations was found nearby chemical plants with 30 % of the examined factories containing most frequently N-nitrosodimethylamine and N-nitrosodiethylamine in ambient air.

This work was supported by Bundesgesundheitsamt, Berlin, FRG

K 10

QUALITATIVE AND QUANTITATIVE DETERMINATION OF NITRATED HYDROCARBONS IN AUTOMOBILE EXHAUST

J. Ries, R. Preussmann & B. Spiegelhalder
Institute of Toxicology & Chemotherapy, German Cancer Research Center, Heidelberg, FRG.

Nitrated hydrocarbons (NHC), in particular nitrated polycyclic aromatic hydrocarbons (NPAH), have been identified in recent years as potent mutagens, some of which are carcinogens in experimental animals. The purpose of this study is to qualititatively and quantiatively determine NHC in automobile exhaust emissions.

Emission measurements were carried out under simulated driving conditions on a motor testing bench equipped with a dilution tube. During these measurements, three different engines (Diesel and Diesel with exhaust recycling) under several driving conditions and other parameters which influence the combustion processes were investigated. A new sampling method was established to collect nitrated phenols. Particle-associated NPAH were collected by high-volume sampling, volatile NHC were sampled on organic adsorption cartridges. The samples were analysed by capillary gas chromatography using a NO-specific chemoluminescence detector (Thermal Energy Analyzer, TEA) and by combined gas chromatography/mass spectrometry using both electron impact (EI) and positive ion chemical ionisation (PICI).

Nitrophenols, methylnitrophenols, dimethylnitrophenols, dinitrophenols and NPAH have been identified and quantified. In most instances 2-nitrophenol is the predominant component observed in concentrations ranging from 0.12 - 95.2 $\mu g/m^3$ undiluted exhaust.

K 11

DEVELOPMENT OF AN ANALYTICAL METHOD FOR PROTEINE PYROLYSIS PRODUCTS BY GAS CHROMATOGRAPHY USING NITROGEN SPECIFIC CHEMILUMINESCENCE DETECTION

M. Meger, R. Preussmann & B. Spiegelhalder
Institute of Toxicology & Chemotherapy, German Cancer Research Center, Heidelberg, FRG.

Protein pyrolysis products constitute an important class of nutritional contaminents found in cooked and charred food. Some of these heterocyclic amines formed by Maillard-type reactions during high temperature food preparation are very potent mutagens when tested with Ames-Salmonella and multipotent carcinogens in long term animal experiments. In order to asses the risk to human health it is essential to quantify the amount to which man is chronically exposed.

A gas chromatograph-chemiluminescence detection method has been developed for the simultaneous measurement of seventeen relevant aminoazaarenes at low ppb levels. Extraction and purification steps are performed as described in literature. Because of their extrem low volatility the method employs derivatization with perfluorinated acylating agents. Up to now we have to devide the known heterocyclics into two classes which require different acylating conditions. Single peaks are identified by gas chromatography/mass spectrometry. For quantification the compounds are introduced via capillary column gas chromatograph into a catalytic heater, where they undergo pyrolysis and oxidation, with release of nitrosyl radicals. The radicals are oxidized with ozone in an evacuated reaction chamber to give electronically excited nitrogen dioxide, which decays back to its ground state with emission of a characteristic chemiluminescence.

The sensitive and selective analysis of nitrogen containing substrates enables us to simplify the isolation scheme with high recovery and thus improve the accuracy of quantification.

K 12

ALKYLATING ACTIVITY IN DAIRY FOODS BEFORE AND AFTER INCUBATION WITH NITRITE

S.E. Shephard, D. Gunz and W.K. Lutz,
Institute of Toxicology, ETH and University of Zürich, CH-8603 Schwerzenbach, Switzerland

The diet contains a large number of constituents which can be nitrosated in the gastrointestinal tract to potentially electrophilic and carcinogenic nitroso compounds. Groenen and Busink recently reported the alkylating potential of a number of food items after incubation with nitrite, using 4-(4-nitrobenzyl)-pyridine (NBP) as a chromogenic nucleophilic reagent. We have continued these investigations. Yoghurt, quark, and buttermilk were incubated with nitrous acid. Nitrate was used for controls. The reaction mixture was loaded on an Extrelut column and lipophilic compounds were eluted with diethyl ether. Ether was evaporated and the non-volatile residue was incubated at 100°C with NBP. At 1.4 mM nitrite, the level of NBP adducts increased 1.4-, 3.7-, and 3.4-fold for the three food items, respectively. Surprisingly, the alkylating potential detected in the *absence* of added nitrite was substantial, in the order buttermilk ≈ yoghurt > quark. At nitrite concentrations found in the normal stomach (5-20 µM), this preformed alkylating potential becomes the predominant risk factor. Compared with the standard precursor carbamoyl alanine and its nitroso derivative, it is estimated that one portion of quark contains the alkylating equivalent of about one µmol nitroso compound of the alkyl urea type. The data indicate that fermentation of milk can result in the formation of products which are stable in acid (in the product and probably also in the stomach) but electrophilic under neutral conditions (as found in the stomach lining cells). The situation is compatible with the nitrosation of alkyl ureas and certain amino acids. Structural analysis will have to show the exact chemical nature of the reactions taking place during this type of food processing.

K 13

Biomonitoring of endogenous piperazine nitrosation

A.R. Tricker [1], R. Kumar [2], M. Siddiqi [3] and R. Preussmann [1]

[1]Institute for Toxicology and Chemotherapy, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg
[2]Department of Pharmaceutical Sciences and
[3]Department of Biochemistry,
University of Kashmir, Srinagar 190006, India

Antihelmintic treatment with piperazine (1,4-diazacyclohexane) for microfilarie parasitism results in the endogenous formation of piperazine-derived N-nitrosamines. The urinary excretion of piperazine-derived N-nitrosamines was determined by biological monitoring of 14 patients receiving 2 g piperazine citrate.

The mean urinary excretion of non-carcinogenic N-mononitrosopiperazine (MNPz) was 27.0 ± 26.7 µg/day (range 0.6-96.0 µg/day). In addition to the expected excretion of MNPz, trace levels of 0.73 ± 0.92 µg/day N,N'-dinitrosopiperazine (DNPz) (range ND-2.8 µg/day) were also found in 7 out of 14 urine samples. N-Nitroso-3-hydroxypyrrolidine (NHPYR), a metabolite of both MNPz and DNPz was detected in 11 out of 14 urine samples at a mean concentration of 1.74 ± 1.72 µg/day (range ND-5.7 µg/day) together with traces of N-nitrosodiethanolamine (NDELA) in two samples at levels of 0.3 and < 0.1 µg/day.

The results show that biological monitoring of urinary NHPYR may be a good indicator of endogenous MNPz formation. In view of the first demonstration of endogenously formed carcinogenic DNPz, piperazine cannot be recommended as a safe precursor for monitoring endogenous nitrosation.

K 14

IDENTIFICATION OF NITROSAMINES FROM THE NITROSATION OF THE ANTIMICROBIAL DRUG HEXETIDINE

P. Mende, R. Preussmann and B. Spiegelhalder
Institute of Toxicology & Chemotherapy, German Cancer Research Center, Heidelberg, FRG.

Hexetidine (1,3-bis(2-ethylhexyl)-5-amino-5-methyl-hexahydropyrimidine) is an antimicrobial drug widely used for therapy of infections in the oral cavity. It contains two tertiary and one primary amino group which are potentially able to undergo reactions with nitrosating agents.

We tested the nitrosatability of hexetidine under the standardized conditions of the "nitrosation assay procedure" (NAP) (10 mM drug, 40 mM nitrite, pH 2-4). Nitroso compounds were isolated by preparative HPLC and identified by mass spectrometry. The main nitrosation product is a heterocyclic dinitroso compound formed after rearrangement of the parent molecule. Five other nitrosamines identified as minor products derive from an open-chain decomposition product of hexetidine.

According to the NAP-test, hexetidine is highly reactive towards nitrosating agents with total nitrosamine yields on the top of a comparative scale on nitrosatability of drugs. Despite this high reactivity, no preformed nitrosamines were detected in commercial hexetidine drug formulations.

However, a nitrosamine formation may occur by endogenous nitrosation of hexetidine in the gastric tract, especially if the drug is swallowed during the application. Data on possible genotoxic properties of the nitrosation products are therefore required for a risk assessment.

K 15

DIETARY AMINES, VOLATILE N-NITROSAMINES AND THE POTENTIAL FOR ENDOGENOUS NITROSATION

B. Pfundstein, B. Spiegelhalder, A.R. Tricker and R. Preussmann
Institute of Toxicology & Chemotherapy, German Cancer Research Center, Heidelberg, FRG.

Primary and secondary amino compounds may react with nitrite within the human body to form direct alkylating species and carcinogenic N-nitroso compounds, respectively. To assess the potential for such endogenous nitrosation reactions from amine precursors in foods amines were determined by a new analytical method using gas chromatography with a thermal energy analyzer allowing specific detection of amines at the 10 µg/kg level.

The results for a dietary survey on nitrosatable amines in the FRG will be presented. The most frequent primary amines in foodstuffs were found to be methylamine, ethylamine, propylamine, iso-butylamine and iso-pentylamine, while the most common secondary amines were dimethylamine, pyrrolidine and piperidine. The presence of these secondary amines in the diet confirm the occasional occurrence of the corresponding preformed N-nitrosamines.

The mean daily intake of primary and secondary amines was calculated to be 37 mg/day/man and 8 mg/day/man, respectively.

Endogenous nitrosation will also be discussed in relation to the presented data on the presence of amines and nitrosating agents (nitrite, nitrate) in saliva, gastric juice, serum, urine and feces.

K 16

PHARMACOKINETICS OF ANTITOXIC AND ANTICARCINOGENIC DITHIOCARBAMATES

A. Scholl, N. Frank
Institut für Toxikologie und Chemotherapie, Deutsches Krebsforschungszentrum, 6900 Heidelberg, Germany

Dithiocarbamtes (DTC) are well known as inhibitors of nitrosodialkylamine induced toxicity and carcinogenicty. Several mechanisms of these effects are discussed: inhibition of metabolizing enzymes, scavenging of ultimate carcinogens or stimulation of detoxifying systems. Recently a protective, antitoxic effect was found when a DTC was administered in combination with different chemotherapeutic agents. Since the mechanisms explaining the anticarcinogenesis are not sufficient to elucidate the antitoxic effects during chemotherapy, more mechanistic investigations should be performed. Here we report the pharmacokinetics of proline- DTC (PDTC), sarcosine-DTC (SDTC) and diethyl-DTC (DDTC) in the rat. The amino acid derived DTC are more hydrophilic than DDTC and show distinct differences in their metabolism. The stability of PDTC and SDTC is higher in vitro and in vivo than that of DDTC. The in vivo metabolism was measured by monitoring CS_2 exhalation and was found to be lowest for PDTC and highest for DDTC. An autoradiographic study showed an enrichment of radioactivity in the kidney and the urether. Whereas DDTC was not excreated, PDTC and SDTC could be detected in urine. DDTC is reported to be neurotoxic and is described to pass the blood brain barrier. In our investigations, PDTC was found not to enter into the brain. This could explain a lower toxicity of PDTC compared to DDTC, and makes PDTC a promising anticarcinogenic and antitoxic agent.

K 17

ACTIVATION OF THE BLADDER CARCINOGEN N-NITROSO-BUTYL-3-CARBOXYPROPYLAMINE BY MITOCHONDRIAL AND MICROSOMAL ENZYMES.

C. Janzowski[1], D. Jacob[1], P. Goelzer[1], I. Henn[2], H. Zankl[2], G. Eisenbrand[1]

[1]Fachrichtung Lebensmittelchemie, [2]Fachrichtung Humanbiologie der Universität Kaiserslautern

N-Nitrosobutyl-3-carboxypropylamine (NBCPA) selectively induces urinary bladder tumors in various animal species. Its mechanism of action is not known yet. α-C-Hydroxylation of NBCPA, detected in microsomes from liver and urinary bladder, is a possible activation mechanism (Janzowski et al., 1989, Toxicology, 59, 195). Metabolic rates of this reaction, however, were extremely low. An attractive alternative for biotransformation is β-oxidation.

Incubation of NBCPA with rat liver mitochondrial fraction (9000xg sediment) yielded N-Nitrosobutyl-2-oxopropylamine (NBOPA) at a rate of 1 nMol x mg protein^{-1} x h^{-1}. In the absence of ATP this β-oxidation reaction is drastically reduced. If S-9 fraction is used instead of mitochondria, NBOPA is not detected.

In contrast to NBCPA, NBOPA is substantially debutylated by rat liver microsomes. Butyraldehyde is also formed by pig urinary bladder microsomes. NBOPA (>15 mM) is genotoxic inducing DNA single strand breaks in Namalva cells on activation with rat liver microsomes. NBOPA (0.3 mM) also induces chromosomal aberrations, micronuclei and SCE in Namalva cells.

These in-vitro data show for the first time that mitochondrial enzymes are relevant for activation of carboxylated nitrosamines to proximate carcinogens.

K 18

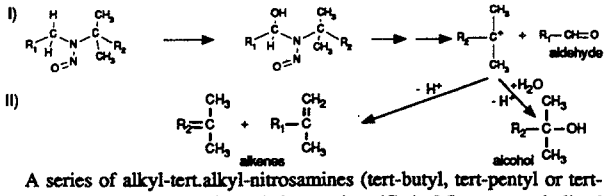
IN-VITRO METABOLISM OF α-TERTIARY BRANCHED N-NITROSAMINES

K.-H. Zimlich, R. Preussmann and B. Spiegelhalder

Institute of Toxicology & Chemotherapy, German Cancer Research Center, Heidelberg, FRG.

Nitrosamines containing an α-tertiary branching neighboured to the N-nitroso group are known to be non-carcinogenic. Reduced alkylating activity of the metabolic intermediates is discussed to be responsible for this effect. We therefore studied the metabolic conversion of α-tertiary branched N-nitrosamines in comparison to carcinogenic N-nitrosamines.

α-Tertiary branched nitrosamines can be hydroxylated only in the α-position of the n-alkyl side chain. This reaction (I) yields an tert. carbonium ion . We found, that this carbonium ion is not only capable to produce the corresponding alcohol through electrophilic reaction, but also generates the analog alkenes by proton elimination (II).



A series of alkyl-tert.alkyl-nitrosamines (tert-butyl, tert-pentyl or tert-hexyl) was synthesized. 7,6 µMol nitrosamines (C=1mM) were metabolized for 30 min. with aroclor-induced rat liver microsomes. Volatile metabolites such as alkenes and alcohols could be identified using headspace gas chromatography. Aldehydes could be identified as 2,4-dinitrophenylhydrazones by HPLC. Representative for nitrosamines containing one α-tert. branching, we list the results for n-alkyl-tert. pentylnitrosamines metabolites (nmoles).

n-alkyl	2-me-1-butene	2-me-2-butene	t-pentanol
methyl-	27	41	316
ethyl-	5.6	5.9	43
propyl-	2.3	2.5	18.2
butyl-	1.2	1.3	12.4
octyl-	1.7	2.4	22.7

K 19

ENZYME POLYMORPHISM IN THE METABOLISM OF METHYLENE CHLORIDE, ETHYLENE OXIDE AND METHYL BROMIDE AND ITS IMPLICATIONS FOR EPIDEMIOLOGY

Barbara Gansewendt, Ricarda Thier, K.R. Schroeder, E. Hallier, H. Peter

The evaluation ot the carcinogenic potency of xenobiotics involves the use of cellular tests for mutagenicity as a primary screening; in the case of a positive result, the substance is applied to laboratory animals in long-term carcinogenicity studies. Definite proof of carcinogenicity to humans can however only be obtained through epidemiologic studies.

Since such human data is very often insufficient due to the limited size of the exposed populations investigated, an evaluation of the carcinogenic potency of a compound is often performed through extrapolation of animal data to humans.

In contrast to the genetically homogenous inbred strains used in animal experimentation, the human population is genetically heterogenous. Over 1000 hereditary enzyme polymorphisms have been identified until today. Such interindividual differences in metabolism can lead to a difference in disposition towards genotoxic effects induced by reactive chemicals, in the worst case to a "high risk" population. This phenomenon cannot be simulated in animal experiments.

In the experiments presented here, several suspected carcinogens were investigated for interindividual differences in metabolism in human blood. ^{14}C-labelled methylene chloride, ethylene oxide and methyl bromide were incubated in separate experiments with human blood samples in a closed exposure system. At regular intervals aliquots were drawn from the samples and the blood was fractionated into its cellular and subcellular components. The distribution of radioactivity in the fractions was determined by liquid scintillation counting. Marked differences in the distribution of radioactivity showed the existence of two distinct subpopulations, which were identical for all three substances investigated. Additional biochemical experiments with methyl bromide in the same exposure system showed that an enzymatic factor was responsible for the interindividual differences in metabolism.

Future investigations on the carcinogenic effect of methylene chloride, ethylene oxide and methyl bromide, especially epidemiologic research, should take the observed enzyme polymorphism in human blood into account.

Institut für Arbeitsphysiologie, Ardeystr. 67, 4600 Dortmund 1, FRG

K 20

INVESTIGATION OF N-NITROSAMINE STRUCTUR-ACTIVITY RELATIONSHIP APPLYING MOLECULAR MODELLING AND *AB-INITIO* CALCULATIONS.

G. Moeckel, B. Spiegelhalder, R. Preussmann &
*W. Heiden, *M. Waldherr-Teschner, *J. Brickmann

Institute of Toxicology & Chemotherapy, German Cancer Research Center, Heidelberg, FRG.
**Institut für Physikalische Chemie, Technische Hochschule Darmstadt*

The chemically stable N-nitrosamines as such are not carcinogenic, but *in vivo* they metabolize to ultimate carcinogens after an enzymatic activation by α-hydroxilase cytochrome P450.

This enzyme-substrate interaction will be studied using the molecular modelling program MOLCAD. It is able to generate a threedimensional representation of molecules and molecular systems which can be manipulated interactively. The solid surface can be used to visualize the electrostatic or the hydrophobic potential. For the calculation of this hydrophobic potential a new algorithm was developed. By using a colour code the local 3D-molecular hydrophobic potential profile is shown on the solid surface, depending on the structural environment and the total hydrophobicity. This enables us to find out the accessibility to the reactive site of the proteine for different groups of nitrosamines.

Additionally *ab-initio* calculations with greater basis sets (6-31g*) are done for estimating geometry and molecular properties like the polarizability of the nitrosamine metabolites. With these theoretical methodes we examine characteristic intermediates of the activation and desactivation pathways, in order to ascertain differences in the behavior of carcinogenic and non-carcinogenic nitroso compounds.

K 21

Metabolism of the tumor promoter 3-O-tetradecanoylingenol in mouse skin and tumor promoting activities of metabolites.

H. Roeser, G. Krauter and E. Hecker
Inst. of Biochemistry, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, FRG

The ingenol monoester 3-O-tetradecanoylingenol (3-TI) is one of the most potent mouse skin tumor promoters of the ingenane type, and is widely used as a standard in biological and biochemical investigations on the mechanism of tumor promotion. Following topical administration of [20-^{3}H]-3-TI to the shaved back skin of female NMRI mice, various metabolites of 3-TI were found in the radioactivity recovered from skin surface, epidermis, and dermis, respectively. They started to appear 1 h after administration, and at 72 h they accounted for about 50 % of the radioactivity recovered from these skin fractions. Long-chain 3-TI-20-acylates were the major metabolites (about 30 %). 5-O-tetradecanoylingenol (5-TI) and long-chain 5-TI-20-acylates, respectively, accounted for about 5 %. 20-O-tetradecanoylingenol (20-TI) was detected only in trace amounts (<1 %). Metabolites more polar than the parent compound were found only in epidermis and dermis, but not in the radioactivity extracted from skin surface. In epidermis ingenol (5 %) was the only polar metabolite detectable, whereas in dermis unidentified polar metabolites (about 5 %) predominated over ingenol (1 %). When tested for tumor promoting activity on the back skin of NMRI mice, long-chain 3-TI-20-acylates as well as 5-TI were almost as potent as 3-TI. 5-TI-20-tetradecanoate - as a prototype of long-chain 5-TI-20-acylates - as well as 20-TI and ingenol were either marginally active as tumor promoters or inactive. Thus it is concluded that the tumor promoting activity of 3-TI may be due to either 3-TI itself or the 3-TI metabolites 5-TI and 3-TI-20-acylates, whereas the 3-TI metabolites ingenol, 20-TI, and 5-TI-20-acylates may be considered products of metabolic deactivation of 3-TI.

K 22

DNA ADDUCTS IN ORAL MUCOSA OF SMOKERS AND NON-SMOKERS

G. Scherer[1], A. Krätschmer[1], H. Hauenstein[2], F. Adlkofer[1]
[1]Analatisch-biologisches Forschungslabor Prof. Adlkofer, München; [2]Klinik und Poliklinik für Mund- Kiefer- Gesichts-Chirugie am Klinikum Minden

Alcohol consumption, use of tobacco products and poor oral hygiene have been identified as major risk factors for oral cavity cancer. Measurement of adducts in DNA from oral mucosa cells could be a suitable method for biomonitoring the target exposure to chemical carcinogens in the oral cavity. Exfoliated oral mucosa cells from users and non-users of tobacco products and alcohol have been used for DNA adduct measurements by the ^{32}P-postlabelling method (1-3). No DNA adducts exclusively related to these habits were reported in these studies. We investigated the DNA adduct patterns by the ^{32}P-postlabelling assay (P1-enrichment version) of oral mucosa, surgically removed during dental operations of 13 patients (5 non-smokers, 16 to 66 years old; 8 smokers, 19 to 50 years old). The DNA adduct patterns were reproducible and characteristic for each individual. No DNA adducts could be found in smokers which were not also present in non-smokers. There was also no indication that age affects the pattern or intensity of the detectable spots. These results together with those from the earlier reports (1-3) let us conclude that exposure to tobacco smoke may lead to formation of DNA adducts, which are not detectable by the ^{32}P-postlabelling method and/or that the oral mucosa is efficiently protected against tobacco smoke carcinogens, probably by action of saliva.

(1) BP Dunn, HF Stich (1986) ^{32}P-Postlabelling analysis of aromatic DNA adducts in human oral mucosa cells. Carcinogenesis 7: 1115-1120.
(2) M Chacko, RC Gupta (1988) Evaluation of DNA damage in the oral mucosa of tobacco users and non-users by ^{32}P-adduct assay. Carcinogenesis 9: 2309-2313.
(3) PG Foiles, LM Miglietta, AM Quart, A Quart, GC Kabat, SS Hecht (1989) Evaluation of ^{32}P-postlabeling analysis of DNA from exfoliated oral mucosa cells as a means of monitoring exposure of the oral cavity to genotoxic agents. Carcinogenesis 10: 1429-1434.

K 23

DETECTION AND QUANTIFICATION OF CARCINOGEN-DNA ADDUCTS BY ^{32}P-POSTLABELING IN THE URINE OF RATS TREATED WITH ARISTOLOCHIC ACID I

R.C. Fernando, H.H. Schmeiser, W. Nicklas*, M. Wiessler
Institute of Toxicology and Chemotherapy and *Central Animal Facility, German Cancer Research Center, 6900 Heidelberg, Germany

Aristolochic acid I (AAI), a nitrophenanthrene derivative, is the main component of the carcinogenic plant extract aristolochic acid. Previous studies showed that AAI is directly mutagenic in the Ames test (Schmeiser et al., 1984). Furthermore, using the ^{32}P-postlabeling assay we found that AAI forms DNA adducts upon metabolic activation *in vitro* and *in vivo* in different organs in the rat (Schmeiser et al., 1988). The two major adducts formed by the reaction of AAI with DNA *in vitro* were characterized as 7-(deoxyguanosin-N^2-yl)-aristolactam I (dG-AAI) and 7-(deoxyadenosin-N^6-yl)-aristolactam I (dA-AAI) (Pfau et al., 1990). This study reports on the detection and quantification of AAI DNA adducts in the urine of rats. Urine samples were collected on dry-ice from male Wistar rats during long-term oral administration of AAI (10mg/kg/day, for 9 weeks), subsequently pooled and purified according to the protocol of Kadlubar and co-workers. DNA was isolated, digested and analyzed by the enhancement techniques of the ^{32}P-postlabeling assay, namely nuclease P1 enrichment or butanol extraction. The resulting autoradiograms indicated that the adduct patterns in DNA from urine were similar to those obtained from DNA isolated from tissues. Adduct levels ranged from 2-10 adducts / 10^9 nucleotides. Count rates of the two predominant AAI adducts were enhanced by butanol extraction about 10-fold when compared with the nuclease P1 treatment. The identity of both adducts was confirmed by cochromatography with eluted spots from *in vivo* adducts by comparing mobilities on PEI-cellulose plates. Microbiological investigations of the urine revealed no gross contamination with bacteria, so that the isolated DNA supposedly originated from exfoliated urothelial cells. This study indicates that ^{32}P-postlabeling analysis can be used to monitor non-invasively the formation of carcinogen-DNA adducts of animals or humans exposed to carcinogens.

K 24

HIGH RESOLUTION CHROMATOGRAPHY OF ^{32}P-LABELLED NUCLEOSIDE 3',5'-BISPHOSPHATE ADDUCTS OF POLYCYCLIC HYDROCARBONS.

Wolfgang Pfau and David H. Phillips

Haddow Laboratories, The Institute of Cancer Research, Sutton, Surrey SM2 5NG, UK

^{32}P-postlabelling is a highly sensitive technique for the detection and analysis of carcinogen-DNA adducts. In order to identify adducts formed by complex mixtures of environmental carcinogens, a chromatographic procedure with higher resolution than the standard ion-exchange thin-layer chromatography on polyethyleneimine-cellulose sheets was required. Here we describe the development of an improved high-performance liquid chromatography (HPLC) method for the separation of ^{32}P-labelled 3',5'-bisphosphates of nucleosides modified by reactive derivatives of carcinogenic polycyclic aromatic hydrocarbons (PAH). Optimum resolution of the major ^{32}P-postlabelled DNA adducts formed by the anti-bay region diol-epoxides of ten PAH was achieved using a phenyl-modified silica gel column with a gradient of methanol in phosphate buffer at low pH and high ionic strength. Use of a radioactivity flow detector coupled to the HPLC apparatus allowed on line detection of subfemtomole quantities of labelled adducts.

K 25

Development of higly sensitive detection of acrolein congener DNA adducts

E. Eder and S. Scheckenbach
Institute of Toxicology, University of Würzburg

α, β-unsaturated carbonyl compounds are ubiquitous environmental pollutants, occurring naturally, in food and as industrial products. They are formed during lipid peroxidation, in lipid and arachidonic acid oxidation and by reactive oxygen species. Acrolein and its congeners are therefore considered to play an important role in mutagenesis and carcinogenesis. The genotoxic activities of these compounds probably depend on their ability to form linear and cyclic adducts with DNA components, in particular, with deoxyguanosine. Unfortunately the detectability of most of these DNA-adducts is very low. In order to clarify the relationship between the interactions of acrolein congeners and mutagenesis and carcinogenesis we are presently developing highly sensitive methods for detection of such adducts, improving the DNA-hydrolysis and analysis methods and adapting the ^{32}P-post-labeling technique using HPLC for the nucleotide analysis. Difficulties arise from the incomplete enzymatic degradation of the modified DNA and, in particular, from the insufficient HPLC separation of the adducts from the unmodified nucleosides. We have achieved a successful separation using Sephadex LH20 gel filtration prior to the HPLC analysis. With this method the modified nucleosides could be analysed, isolated and identified much more readily with improved sensitivity, since the unmodified nucleoside peaks and those of the adducts no longer overlap.

K 26

INVESTIGATION OF THE POTENTIAL FOR DNA BINDING OF STYRENE-7,8-OXIDE IN RAT AND MOUSE

S. Cantoreggi and W.K. Lutz,
Institute of Toxicology, ETH and University of Zürich, CH-8603 Schwerzenbach, Switzerland

Styrene oxide, the main intermediate metabolite of styrene, induces hyperkeratosis and tumors in the forestomach of rats and mice upon chronic administration in the diet. The aim of this study was to investigate whether DNA binding could be responsible for the carcinogenic effect observed. [7-^{3}H]-Styrene-7,8-oxide was administered by oral gavage in corn oil to male Sprague-Dawley rats at 2 dose levels (1.6 or 220 mg/kg). After 4 or 24 hours, forestomach, glandular stomach, and liver were excised and DNA was purified extensively. At the 4-hour time point, the DNA radioactivity was below the limit of detection in all samples. Expressed in the units of the Covalent Binding Index, CBI = (μmol adduct per mol DNA nucleotide)/(mmol chemical administered per kg body wt.), the DNA-binding potency was below 3, 2, and 0.3, in the forestomach, glandular stomach, and liver, respectively. At the 24-hour time point, DNA isolated from both parts of the stomach (but not from the liver) was slightly radiolabelled. Metabolic degradation of [7-^{3}H]styrene oxide can result in the formation of tritiated water. It is therefore assumed that the DNA radioactivity in the gastrointestinal tract represented biosynthetic incorporation of radiolabel into newly synthesized DNA. In a second experiment, [7-^{3}H]styrene-7,8-oxide was administered by intraperitoneal injection to male B6C3F1 mice. Liver DNA was analysed after 2 hours. No radioactivity was detectable at a limit of detection of CBI<0.6. It is concluded that the reactivity of styrene oxide with nucleic acids is not detectable *in vivo* at a low detection limit. This finding agrees with the relative stability of styrene oxide in the blood (half life ≈ 20 min). Upon comparison with other carcinogens, the maximum possible CBI values render a purely genotoxic mechanism of tumorigenic action unlikely.

K 27

COMPARISON OF THE GENOTOXIC POTENCY OF ETHYLENE OXIDE AND VINYL CHLORIDE ON THE BASIS OF DNA ADDUCTS

Monika Leutbecher, H. Peter, H. Ottenwälder, U. Föst

Ethylene oxide is an air pollutant emitted by various sources like exhaust fumes and cigarette smoke. In addition, it is metabolically formed in mammals after inhalation of ethylene, which is also produced in combustion processes and found as a phytohormon. The carcinogenic potency of ethylene oxide has clearly been proven in animal studies.

Vinyl chloride, a proven human carcinogen, is one of the most widely produced monomers for plastics. No natural sources of vinyl chloride are known. The substance is also produced from air pollutants like trichloroethylene and perchloroethylene in soils by anaerobic bacteria leading to contamination of drinking water.

Male Sprague-Dawley rats were exposed to either ethylene oxide or vinyl chloride (3 doses/experiment; 3 animals /dose). Liver-DNA was isolated by phenol extraction and the amount of 7-(2-hydroxyethyl)-guanine in the case of ethylene oxide exposure and 7-(2-oxoethyl)-guanine in the case of vinyl chloride exposure were detected by gas chromatography/mass spectrometry. Chemical binding indices (CBI) according to Lutz were calculated from these data as well as from data published earlier by other groups. A comparison of the CBI's of ethylene oxide and vinyl chloride calculated from 7-alkylated guanines in liver DNA of pretreated rats showed an approximately 70-fold higher genotoxic risk for vinyl chloride than for ethylene oxide.

In additional experiments, the distribution pattern of ethylene oxide in the compartments of human blood was investigated in vitro using ^{14}C-labelled substance. It was found that an enzymatic turnover of ethylene oxide in human blood is probably subjected to an enzyme polymorphism, which may influence a binding of the substance to hemoglobin.

In order to evaluate the additional carcinogenic risk from endogenously produced ethylene oxide, lymphocyte DNA from non-exposed animals and humans was isolated and the amounts of 7-(2-hydroxyethyl)-guanine was determined as described above. Adducts specific for ethylene oxide can in part stem from endogenous production. The high level of adducts in non-exposed rats and humans cannot be explained by this production alone, so that exogenous sources such as an ubiquitous air pollution must be taken into consideration.

Institut f. Arbeitsphysiologie, Ardeystr. 67, D-4600 Dortmund 1

K 28

MUTAGENIC ACTIVITY IN E. COLI BY O^{6}-N-BUTYLGUANINE AND O^{6}-N-OCTYLGUANINE BUILT INTO A UNIQUE SITE IN A PLASMID

P.M. Baumgart, H.H. Schmeiser, H.-C. Kliem, S.Data, M. Wiessler
Institut für Toxikologie und Chemotherapie, Deutsches Krebsforschungszentrum, 6900 Heidelberg, Germany

The reaction of alkylating agents with DNA results in the formation of a diverse population of alkyl-DNA adducts, the processing of which is believed to lead to mutations and possibly cancer. One of the best investigated groups of alkylating agents are the N-nitrosamines. In contrast to other dialkylamines di-n-butylnitrosamine (NDBA) is not a typical livercarcinogen in the rat, but also induces tumors of the urinary bladder. NDBA was shown to occur in the atmosphere of factories of the rubber industry (Spiegelhalder et al., 1983). Reactions with N-nitroso-n-butylurea or NDBA lead to butylation of DNA. Here we report on our studies with O^{6}-n-butylguanine and O^{6}-n-octylguanine which we additionally included for comparison to a long-chain derivative. Using site directed mutagenesis we examined the mutagenic activity in E. coli of both modified guanines located at a unique site in a plasmid. The modified 10mer oligonucleotides were prepared on a DNA synthesizer and characterized by HPLC, nucleoside composition analysis and ^{32}P-postlabeling. These 10mers were ligated into pUC9 by "shot-gun" gene synthesis using another 10mer and 20mer with overlapping sequences. After cloning in E. coli BMH 71-18, mutants were identified by digestion with XhoI or PstI and sequenced by the dideoxy-method. O^{6}-n-butyldG situated at a unique site in pUC9 showed mutagenic activity by targeted mutations, all of which being GC→AT transitions. In contrast O^{6}-n-octyldG leads to 70% GC→AT transitions and to 30% GC → TA transversions. We assume that the mutagenic activity caused by O^{6}-n-butyldG results from mispairing, whereas for O^{6}-n-octyldG mispairing is partially inhibited sterically by the bulky n-octyl group. The 30% GC→TA transversions could be the result of insertion of adenine opposite to a non-coding lesion, namely O^{6}-n-octyldG.

K 29

EFFECT OF DNA ALKYLATION BY NITROSOUREAS ON SEMICONSERVATIVE DNA SYNTHESIS BY PROCARYOTIC DNA POLYMERASE

C. Pfeiffer, A. Fuchs, G. Eisenbrand
Universität Kaiserslautern, FR Lebensmittel-chemie/Umwelttoxikologie, P.O.B. 3049, D-6750 Kaiserslautern, FRG

We have incubated M13 mp18 phage DNA with a series of homologue alkylnitrosoureas to study the effect of alkylation on the propensity of single stranded DNA to act as a template for DNA synthesis using E. coli polymerase Klenow fragment (Kf polymerase). Treatment with N-methyl-N-nitrosourea (MNU; 1-20 mM) resulted in a concentration dependent induction of DNA polymerase stops, 41/128 stop signals being clearly detectable. The sequencing gel revealed methylation predominantly one base 3'to template adenine residues (26/41), with some modification occuring also at sites 3'to template guanine (8/41) and cytidine (6/41).
Treatment with longer chain nitrosoureas (ethyl- to n-butyl; 1-50mM) resulted in much weaker inhibitory effects on polymerase-mediated chain elongation with respect to frequency as well as intensity of stop signals. Moreover, no clear concentration dependency was detectable. Introduction of polymerase stops appeared to shift, however, with increasing chain length from one base 3'of adenine to one base 3'of guanine. In conclusion, the extent of induction of DNA polymerase stops mainly reflects the differential alkylating potency of homologuous nitrosoureas rather than the size of the alkylgroup transferred.

K 30

THE MICROGEL SINGLE CELL ASSAY: A RAPID AND SENSITIVE TECHNIQUE TO ANALYSE DNA DAMAGE IN PRIMARY RAT CELLS FROM VARIOUS ORGANS.

P. Schmezer[1], F. Kuchenmeister[1] and B.L. Pool-Zobel[2].
1: Institute for Toxicology and Chemotherapy, German Cancer Research Center, INF 280, W-6900 Heidelberg,
2: Institute for Hygiene and Toxicology, Federal Research Center for Nutrition, Engesserstr. 20, W-7500 Karlsruhe, F.R.G.

The microgel single cell assay developed by Ostling and Johanson (1984, Biochem. Biophys. Res. Commun. 123:291) and modified by Singh et al. (1988, Exp. Cell. Res. 175:184) is an electrophoretic technique to detect DNA single-strand breaks and alkali labile sites in individual cells. We used this approach to analyse DNA-damaging compounds in primary cells derived from rat liver, stomach, blood and nasal mucosa in vitro and ex vivo. For this, the treated cells were embedded in agarose gels on microscope-slides, lysed and the remaining DNA was treated with alkali. Subsequently, a 20min. electrophoresis (25V, 300mA) was performed and the neutralized slides stained with ethidium bromide. As a quantitative measure of the induced DNA-damage, the migration patterns of the DNA in the agarose gels were analyzed by fluorescence microscopy coupled with an image analyzer. So far e.g. the evaluation of 100 cells/slide/compound concentration or dose have revealed potent in vitro genotoxic effects of methylnitrosourea in tumor cells and nitrosodimethylamine (NDMA) in hepatocytes. Furthermore, methylnitrosoguanidine, NDMA, and a tobacco specific nitrosamine were genotoxic ex vivo in cells of various organs. In comparison to the well-established procedure of the alkaline filter elution, this technique showed a comparable sensitivity but was much less time-consuming. Moreover, it has the distinct advantage that very few or even single cells may be monitored.

K 31

SINGLE CELL ANALYSIS OF CARCINOGEN-INDUCED DNA SINGLE STRAND BREAKS BY IN SITU NICKTRANSLATION

U.M. Liegibel, P. Schmezer, *B.L. Pool-Zobel
Institute for Toxicology and Chemotherapy, DKFZ, Heidelberg.
*Institute for Hygiene and Toxicology, Federal Research Center for Nutrition, Karlsruhe.

In order to assess toxicokinetic parameters and other mechanisms of genotoxic carcinogens it is useful to investigate compound-induced DNA single strand breaks in a variety of primary cells of the rat *in vitro* as well as *in vivo*. For this, we need methods that can detect DNA damage in even a very small number of cells. One potential technique is the visualization of DNA-strand breaks ("nicks") by *in situ* - nick translation (NT). The slides with the fixed cells are incubated with the repair enzyme E. coli DNA Polymerase I and the nucleosidetriphosphates ATP, CTP, GTP and [^{3}H]TTP for 10 minutes at 21°C. Incorporation of [^{3}H] into DNA is detected by autoradiography and quantified by counting the grains per nucleus (Anai H. et al., Cancer Letters 40, 33, 1988). To asssess the sensitivity of this new method we have performed some experiments after a one hour incubation of primary hepatocytes with the test substance and compared the results to those obtained by the well established technique of alkaline filter elution (AE). As test compounds we used different N-nitrosamines most of which need metabolic activation for genotoxic activity. Results show that NT is able to detect single strand breaks in primary cells. N-Nitrosodimethylamine induced DNA damage at approximately the same concentrations in both systems. N-Nitrosomethylbenzylamine induces detectable single strand breaks in hepatocytes at 10 µmoles/ml in NT, whereas 1 µmol is sufficient for AE. For the tobacco-specific nitrosamines NNN and NNK no effects can be measured with NT up to 25 µmol/ml but NNK induces DNA-single strand breaks at 6.25 µmoles measured with AE. Whereas the number of cells needed for one experiment is much lower for NT than for AE (1-2 x 10^5/slide for NT; 1-5 x 10^6/ filter for AE), NT is somewhat less sensitive.

K 32

A NICK TRANSLATION ASSAY AS A SPECIFIC SCREENING METHOD FOR POTENTIAL INITIATING AGENTS OF SKIN

L.Steeb, H.Friesel, Th.Schneider and E.Hecker, Institute of Biochemistry, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, F.R.G.

Polycyclic aromatic hydrocarbons (PAH) such as 7,12-dimethylbenz-[a]anthracene (DMBA) are efficient initiators of mouse skin in vivo and transforming agents in fibroblasts in vitro. These effects are irreversible and may depend on initial covalent binding to DNA. One of the possible consequences of such binding, the induction of DNA single strand (ss) breaks, was studied with Balb c 3/T3 cells in an in vitro nick translation assay up to 24 hours after exposure. For this purpose the cells were maintained and treated in 24-well culture plates for high sample throughput. Permeabilization was achieved by treatment with lysolecithin and monitored by trypan blue. Care was taken that the cells remained adherent throughout the nick translation reaction as initiated by addition of DNA polymerase I, ^{3}H-dCTP and unlabelled dNTPs. DMBA (400nmole), the most potent initiator of skin in vivo, was compared to equimolar and equicytotoxic doses of either the PAH 9,10-dimethylanthracene (DMA) or the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA).
The incorporation of ^{3}H-dCTP in nicked DNA (values for tested compounds corrected by subtraction of solvent control values) obtained with DMBA show that following a decrease during the first 4 hours the ss-breaks increase to a maximum at 7 hours, whereas DMA was inactive even at equicytotoxic doses. Also the tumor promoter TPA causes no detectable ss-breaks. Therefore nick translation to measure ss-breaks may be a specific in vitro assay for potential initiating activity, since it can efficiently discriminate between DMBA (a tumor initiator) and its inactive derivative DMA or TPA (a tumor promoter). This working hypothesis will be investigated using a large number of DNA-binding, initiating and non-initiating PAH.

K 33

ACTIVATING MUTATIONS AT CODON 61 OF THE C-HA-RAS GENE IN TUMORS OF RATS AND MICE INDUCED BY ARISTOLOCHIC ACID

H.H. Schmeiser, H.R. Scherf and M. Wiessler

Institute of Toxicology and Chemotherapie, German Cancer Research Center, 6900 Heidelberg, Germany

Aristolochic acid I (AAI), a nitrophenanthrene derivative, is the major component of the carcinogenic plant extract aristolochic acid, which has been used as a medicine since antiquity. Long term oral administration of AAI to male Wistar rats induces multiple tumors, mainly in the forestomach. We previously reported (Schmeiser et al., 1990, Cancer Res. 50, 5464-5469) on the detection of AT→TA transversion mutations in codon 61 of the c-Ha-ras gene (CAA to CTA) in 93% of the forestomach tumors analyzed. Here we applied PCR and mutation detection by selective oligonucleotide hybridization to 5 µm sections of formalin-fixed paraffin-embedded tissue. Paraffin blocks of forestomach squamous cell carcinomas from rats containing adjacent carcinoma and histologically normal tissue were cut and manually separated. DNA obtained from these histologically separated regions was amplified and analyzed. AT→TA transversions in codon 61 of the c-Ha-ras gene were observed in the carcinoma portions, but not in the adjacent normal tissue.

Moreover we screened 11 forestomach and 12 lung tumors of female NMRI mice induced by aristolochic acid (80% AAI;20% AAII) for c-Ha-ras gene mutations in codon 61 by the same procedure. Only 1 in 11 forestomach sections and 3 in 12 lung sections were successfully amplified. Identical AT→TA transversions were detected in the squamous cell carcinoma portion of the forestomach section as well as in one adenocarcinoma portion of one lung section. These results indicate that aristolochic acid activates ras genes in both rat and mouse tumors at deoxyadenosine residues.

K 34

MUTATIONS IN THE c-Ha-RAS GENE OF CARCINOGEN- AND TUMOR PROMOTER-INDUCED MOUSE LIVER TUMORS

A. Buchmann[1], R. Bauer-Hofmann[2], J. Mahr[2] and M. Schwarz[2]

[1]Institute of Toxicology, University of Tübingen, D-7400 Tübingen
[2]Institute of Experimental Pathology, German Cancer Research Center, D-6900 Heidelberg

It is well documented that different strains of rodents show characteristic differences in both, their spontaneous rate of liver tumor formation and their susceptibility towards hepatocarcinogenic and/or tumor promoting agents. Although the molecular basis for these differences in susceptibility to hepatocarcinogenesis is largely unknown, recent results from our laboratory have indicated that mutational activation of the c-Ha-ras gene may be a major factor contributing to liver tumor development in sensitive but not in insensitive rodent strains. By analysis of liver tumors from various mouse strains which occurred either spontanously or after carcinogen-treatment, we were able to detect mutations at codon 61 of the c-Ha-ras gene in 30-60% of spontaneous or carcinogen-induced liver tumors of different sensitive mouse strains (e.g. C3H/He, B6C3F1 and CF1 mice) but not in liver tumors of the comparatively insensitive C57BL/6J mouse. To further extend our findings, C3H mouse liver tumors induced by non-genotoxic tumor promoters such as phenobarbital or dieldrin were analyzed. Although treatment with these compounds more than doubled the number of liver tumors per animal as compared to untreated controls, the frequency of tumors with c-Ha-ras mutations was lowered from 60% in controls to approximately 25% in promoter-treated groups. This finding suggests that c-Ha-ras mutations within promoter-induced liver tumors represent background mutations not related to treatment and, secondly, that tumor promoters also confer a selective growth advantage on those initiated hepatocytes that do not contain an activated c-Ha-ras gene.

K 35

SOLITARY CANCEROGENESIS BY 7,12-DIMETHYLBENZ[a]ANTHRACENE (DMBA) IN MOUSE EPIDERMIS: THE INFLUENCE OF DOSE, DOSE-FREQUENCY AND OF THE TUMOR PROMOTER TPA

H. Friesel, R. Schmidt, F. Rippmann, B. Steinbauer, and E. Hecker. Institute of Biochemistry, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, F.R.G.

The solitary cancerogenic effect of DMBA was studied, varying the intervals between repeated epicutaneous administrations to the back skin of female NMRI mice and, concomitantly, the partial doses d in such a manner that they added up to equal total doses D over a given period of time. The total number of benign and that of malignant tumors was recorded weekly. The total dose D = 800 nmol was highly cancerogenic when administered as d = 18 nmol twice weekly during 22 weeks and observed up to 38 weeks; under these conditions, D = 800 nmol given as d = 54 nmol every 10 days was marginally active and d = 100 nmol every 4 weeks showed no effect. 44 x d = 18 nmol proved to be even more effective than 5 x d = 1000 nmol. Similarly performed lifelong continuous applications led to drastic increases in latency periods at longer intervals as well as decreases in the ratio of carcinomas vs papillomas. These results suggest a partial reversibility of cancerogenic effects by DMBA, possibly due to a promoting activity requiring a constant stimulus. Alternatively, insufficient DNA repair at short intervals may lead to cumulation of genotoxic lesions. Twice weekly co-administrations of DMBA and TPA did not increase the number of carcinomas but led to a high number of papillomas, similar as with initiation by a single dose of DMBA followed by promotion with TPA. This shows that TPA can affect epidermal cell populations different from those involved in solitary cancerogenesis.

K 36

Effects of Dietary Restriction on Mammary Tumorigenesis, Sexual Hormones and Hormone Receptors

P. Zlfu, B. Bunk, W. Hefter, K. Klinga and M.R. Berger

Institute of Toxicology and Chemotherapy
German Cancer Research Center, Heidelberg, FRG

Female Sprague–Dawley rats were injected i.v. with 25mg N–methylnitrosourea/kg on day 50 of life to induce mammary carcinoma, and divided into 6 dietary groups: 50, or 35 kcal/day with 45, 35 and 25% (energy%) fat, respectively, were fed for 27 ± 1 weeks. In accordance to a previous study from our laboratory, tumor burden was reduced in the low calorie groups, independently of the fat levels. Among the high calorie groups, tumor number and weight were significantly higher in the 35% than in the 25% fat group, but both levels showed no difference to the 45% fat group. By comparing all low calorie groups versus high calorie groups, both plasma estradiol (E2) and testosterone (T) levels at the stage of diestrus were lower in the low calorie group (E2: $p<0.05$ and T: $p<0.01$). Furthermore, an elevated T/E2 ratio in the low calorie group was found ($p<0.05$). No relationship of either E2 or T levels to fat levels could be observed. Free estradiol and progestrone receptor level in tumor cytosol showed no difference among the groups. Lower mean cytosolic androgen receptor level and higher dissociation constant were observed in tumor tissue of rats fed 35 kcal/day compared to the 50 kcal group ($p<0.05$), which could be a response to the elevated plasma T/E2 ratio found in the calorie restricted group.

In conclusion, calorie restriction inhibits mammary tumor development, reduces plasma sexual hormone levels independently of dietary fat content, and elevates plasma T/E2 ratio; the latter might interfere with tumor burden.

K 37

DETERMINATION OF THE INITIATING AND PROMOTING ACTIVITY OF XENOBIOTICS IN RAT LIVER

M. Schwarz[1], A. Buchmann[2], L.W. Robertson[3], G. Luebeck[4] and S. Moolgavkar[4]

[1]Projekt "Tumorpromotion in der Leber", Deutsches Krebsforschungszentrum, Heidelberg
[2]Institut für Toxikologie der Universität Tübingen
[3]Grad. Center for Toxicology, University of Kentucky, Lexington, USA
[4]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, USA.

The improvement of methods used to evaluate possible risks associated with exposure of humans to environmental compounds is one of the challenging tasks of toxicology. Recently developed biologically-based methods for cancer risk assessment seem to be very suitable in this respect both for inter-species and for low-dose extrapolation of experimental data.
Compounds that increase the risk of cancer may exert their activity on the stages of initiation and/or promotion and/or progression. On the basis of theoretical considerations regarding the underlying mechanisms of action, characteristic differences can be expected to exist in the dose-response characteristics between initiating and promoting activities of such compounds.
We have now determined the formation of enzyme-altered foci in livers of rats treated continuously with various hepatocarcinogens and in addition, in animals that were given various polyhalogenated biphenyls either alone or in combination with an initiating dose of diethylnitrosamine. Differences in the effects of the various test compounds on the number and size distribution of foci were taken as indicators of differences in their initiating and promoting activity, respectively. In addition, foci data obtained with selected compounds were quantitatively analyzed within the framework of the model developed by Moolgavkar and colleagues. The outcome of these studies clearly indicates that this latter approach represents a very valuable tool for the quantitative analysis of such data and may significantly improve quantitative cancer risk assessment.

K 38

Hormone-dependency, growth-rate and morphology of mammary tumours in SD-rats treated with NMU

Torsten, U., Kowalczyk-Wagner, M., Weitzel, H.
Dept. of Gynecology and Obstetrics
Klinikum Steglitz, Free University Berlin,
Hindenburgdamm 30, W - 1000 Berlin 45
FRG

SD-rats were deprived in estrogen by operation or by application of anti-estrogens at different stages in the development of breast cancer. One group received ovariectomy and another group daily application of the anti-estrogen TAMOXIFEN simultaneously with the chemical induction by the carcinogen NMU. In the ovariectomized group 62,5 % of the animals showed no tumours compared to 80 % of those which got TAMOXIFEN. In another series one group was ovariectomized and another received TAMOXIFEN after appearance of tumour nodules; ovariectomy led to a 57 % complete remission, whilst TAMOXIFEN at this stage of carcinogenesis did never show a complete or partial remission.

K 39

Expression of oncogenes in xiphophorine tumors of different etiology

Ch. Zechel, H. Peters, and F. Anders

Melanoma in Xiphophorus is mediated by a tumor gene complex (Tu-complex). Melanoma develops after deregulation of the Tu-complex a) in the germ line by mutation, b) in a pigment cell precursor by somatic mutation, and c) following the impairment of a tumor preventing block of cell differentiation by tumor promoters.

We studied the inheritance, structure and expression of several genes in fish strains sensitive for each of these kinds of tumor etiology.

Southern blot analysis and sequencing showed that a c-erbB-related gene (x-erbB*) is part of the Tu-complex. Expression and overexpression of x-erbB* was detected in spontaneous tumors as well as in initiator- and promoter-induced tumors. The highest expression was found in fast growing spontaneous and promoted melanomas. A second type of erbB-related gene (x-erbB) which probably encodes the common epidermal growth factor receptor (EGF-R) is expressed at a low level in all three kinds of tumors. Genes related to the human genes coding for the plateled derived growth factor (PDGF) and its receptor (PDGF-R) were identified in Xiphophorus by Southern Blot analysis and sequencing. Expression of x-pdgf was detected in approx. half of the spontaneous melanomas and fibrosarcomas. The x-pdgf-r is expressed in all tumors studied and is slightly overexpressed in spontaneous melanomas and fibrosarcomas. Expression of three types of identified x-erbA-genes that probably code for proteins of the thyroid hormone receptor family was undetectable in the majority of the tumors studied.

So far, only the x-erbB* appears to be involved in the initial steps leading to melanoma and possibly to certain other tumors. The role of x-erbB, pdgf, pdgf-r, and x-erbA in tumor formation remains unclear. Supported by University of Giessen, DFG, and UBA.

K 40

AAF AND LIVER CANCER IN RATS

**S. Ambs, T.Kirchner and H.-G. Neumann
Institut für Toxikologie und Institut für Pathologie, Universität Würzburg**

2-Acetylaminofluorene (AAF) is a complete rat liver carcinogen (initiator and promotor). We studied the role of cytotoxicity for the promoting activity of AAF. Male Wistar rats were chronically fed 0.02% AAF in the diet for up to 16 weeks. The livers were analysed histologically. The following changes are seen in male rats: After 4 weeks the first GST-P positiv foci are detectable. Thereafter the area of these foci increases exponentially. Between the third and fifth week a new cell population appears in oxygen rich regions with a periportal accentuation. This cell population (gamma-GT positiv, ductular morphology similar to small bile ducts) generates septa encircling the centrilobular areas. The appearance reminds of the swine liver and is especially visualized by silver staining of reticulin fibers. Between week 8 and 12 livers become cirrhotic.
The origin of these duct-like cells is unknown. One possible explanation is that AAF initiates transformation of hepatocytes to duct-like cells by a mito-inhibitory or hypoxic effect in the oxygen rich regions. Advanced stages exhibit multiple necrosis of hepatocytes and the development of cirrhosis, in which the disturbance of microcirculation may produces selective pressure on hepatocyte proliferation. As a first step, we postulate the interaction of AAF metabolites with mitochondria of hepatocytes. Mitochondria in the periportal area are generally larger, have a higher oxygen turnover, and enzyme activities are different from mitochondria of the centrilobular area.
Investigations on isolated mitochondria are in progress.

K 41

G6PDH AND LDH AS MARKERS FOR FOCI INCIDENCE AFTER TREATMENT WITH DEN AND CLOPHEN A 50.

[1]C. Einig, [1]E. Eigenbrodt, [2]D. Oesterle, [2]E. Deml, [3]G. Weisse, [4]U. Gerbracht

The rat liver foci bioassay is widespreadly used as an in vivo test system for the identification of chemically induced hepatocarcinogenesis. Following the two stage model of carcinogenesis which implicate the treatment with an initiator and a promotor, the rats received once 10 mg/kg b.wt of diethylnitrosamine (DEN) which is a strong liver-carcinogenic chemical. After 7 days the animals were treated twice a week with the promoter Clophen A 50 (10 mg/kg b.wt) for the following 10 weeks. After the experiment the rats were killed and the liver cryostat slices were stainded histochemically. The enzymes lactate-dehydrogenase (LDH) and glucose-6-phosphate-dehydrogenase (G6PDH) of the carbohydrate metabolism were used for the endpoint determination. The most of the foci were recognized in the animal group which had received the complete treatment schedule in contrast to the groups which were treated only with DEN or Clophen A 50. Also the mean area of the foci, the total area of the foci /cm^2 of the liver slice and the relative size distribution of the foci indicate that in the group which received the complete schedule the islands were significantly increased.

1 Vet. Biochemie Universität Gießen Frankfurterstr. 100 6300 Gießen
2 GSF Institut für Toxicol. Neuherberg
3 Merck, Institut für Toxikologie, Darmstadt
4 Pharmbiodyn Denzlingen

K 42

Detection of glucose-6-phosphate dehydrogenase m-RNA in hepatocellular tumours and preneoplastic liver lesions of the rat

H. Stumpf,E. Filsinger und P. Bannasch

Institut für Exp. Pathologie, Deutsches Krebsforschungszentrum Heidelberg

The appearence of hepatocellular carcinomas, induced in rats with N-nitrosomorpholine, is preceded by a series of preneoplastic liver lesions, leading from glycogen-storing-foci (GSF) to mixed cell foci (MCF) and basophilic foci (BCF), which share a number of alterations in enzyme activity. One of the most prominent changes is the increase in the activity of the glucose-6-phospate dehydrogenase (G6PDH), the key enzyme of the pentose-phosphate-pathway.
Using Northern-Blot-Analysis we were able to show increased levels of G6PDH-transcripts in 9 out of 10 hepatocellular carcinomas tested.To avoid dilution effects we used "in-situ"-hybridization to detect G6PDH-transcripts in focal lesions. GSF and the glycogen storing cells of MCF showed no detectable elevation in G6PDH m-RNA levels, though they were positive in enzyme activity. The basophilic cells of MCF and the BCF exhibited a strong increase in G6PDH-RNA. Three carcinomas that served as positive controls showed an even stronger elevation of the transcript.

K 43

EFFECT OF THE HEPATOCARCINOGEN 2-ACETYLAMINOFLUORENE AND ITS NONCARCINOGENIC ISOMER 4-ACETYLAMINOFLUORENE ON THE LEVEL OF PLASMA MEMBRANE RECEPTORS AND ON THE ACTIVITY OF CARBOHYDRATE-METABOLIZING ENZYMES IN RATS

W. Muster and P. Cikryt, Inst. für Toxikologie der Universität Würzburg

The aromatic amines 2-acetylaminofluorene (2AAF) and 4-acetylaminofluorene (4AAF) differ in their toxic and carcinogenic properties. 2AAF is a potent hepatocarcinogen in the rat and has tumor-promoting properties. In contrast to 2AAF, the isomer 4AAF is regarded as a noncarcinogen or at least as a less potent carcinogen than 2AAF. It is established that the activity of carbohydrate-metabolizing enzymes changes during the carcinogenic process, but the relationship between these changes and tumor promotion is not clear. For a number of diverse chemical tumor promotors, it is hypothesized that cellular receptors, e.g. plasma membrane receptors, may be involved. The objective of this study was to compare the effect of the complete carcinogen 2AAF on carbohydrate-metabolizing enzymes and on plasma membrane receptors with the effect of the putative noncarcinogen 4AAF. Male Wistar rats were treated with 0.02 % 2AAF or 0.1 % 4AAF in the diet for 2, 7 or 21 days. The level of epidermal growth factor receptor (EGFR) of plasma membranes was down-regulated only by 2AAF after only 2 days of treatment and did not then recover. Microsomal EGFR content was reduced significantly by 2AAF and 4AAF. Cytosolic protein kinase C activity was significantly reduced by 2AAF after 21 days of treatment and by 4AAF after only 7 days of treatment . Changes in the activity of carbohydrate-metabolizing enzymes were as follows: the gluconeogenetic enzyme phosphoenolpyruvate carboxykinase was reduced to 28 % and 46 % of control values by 2AAF and 4AAF, respectively, after 21 days of treatment. Glucose-6-phosphatase activity was reduced by both aromatic amines after 7 days of treatment. Hexokinase activity remained unchanged. Only 2AAF treatment increased glucose-6-phosphate dehydrogenase activity 5-fold after 21 days. The results of this study clearly demonstrate that some of the observed effects are only evoked by the complete carcinogen 2AAF. Other changes are also observed with the noncarcinogen 4AAF. The importance of these modifications for the process of tumor promotion remains to be elucidated.

K 44

EXPRESSION AND REGULATION OF PHENOL UDP-GLUCURONOSYL-TRANSFERASE IN HEPATOCYTE CULTURES, HEPATOMA (H4IIE) CELLS AND IN UNTRANSFORMED AND TRANSFORMED RAT LIVER EPITHELIAL CELLS

P. Münzel, E. Röhrdanz and K.W. Bock
Institute of Toxikology, University of Tübingen, W-7400 Tübingen, FRG

UDP-Glucuronosyltransferases (UGTs) are involved in detoxication and elimination of endobiotics and of a vast array of xenobiotics including carcinogens. One isozyme of this supergene family, termed phenol UGT, is expressed at a low level in hepatocytes, but inducible by 3-methylcholanthrene (MC)-type inducers and by multiple other factors. The enzyme levels appears to be high in H4IIE hepatoma cells and in rat liver epithelial cells (RLEs). To study mechanisms responsible for constitutive expression of the isozyme, a phenol UGT-selective DNA probe was used. Two oligonucleotide primers, prepared according to its cDNA, and isolated rat liver DNA were used to synthesize a 280bp fragment (nucleotide 71-350) by the polymerase chain reaction. In the presence of 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD) phenol UGT mRNA level was increased 10-fold in primary hepatocytes. Persistently increased expression (7-fold) was observed in H4IIE cells and expression was further inducible by TCDD. Regulation of phenol UGT was also studied in RLEs (McMahon et al. 1986, Cancer Res. 46, 4665). Phenol UGT expression depended on cell density and passage number. Aflatoxin B1-transformed RLEs and RLEs of higher passages showed even higher phenol UGT mRNA levels than RLEs of low passage number.
The results suggest that (in contrast to primary hepatocytes) H4IIE cells, RLEs and transformed RLEs show high persistent expression of phenol UGT, a behaviour which is also found at cancer prestages (hepatocyte foci and hepatocyte nodules). The cell lines may be useful to elucidate mechanisms responsible for high constitutive expression of the isozyme.

K 45

BIOLOGICAL ACTIVITIES OF MIXTURES OF POLYCHLORINATED DIBENZO-P-DIOXINS (PCDDs) AND THEIR CONSTITUENTS IN HUMAN HEPG2 CELLS

H.-P. Lipp, T. Wiesmüller*, H. Hagenmaier*, D. Schrenk and K.W. Bock
Institute of Toxicology and *Institute of Organic Chemistry, University of Tübingen, W-7400 Tübingen, FRG

Polychlorinated dibenzo-p-dioxins (PCDDs) are environmental contaminants which cause a variety of toxic symptoms including body weight loss and thymic atrophy in rats. A good correlation has been reported between the toxic potency of various PCDDs in vivo and their potency to induce P450IA1 activities in the H4IIE rat hepatoma cell line (Safe S., Ann. Rev. Pharmacol. Toxicol. 26, 371-399, 1986). In a previous study it was found that inducing potencies of PCDDs in H4IIE cells and in rat hepatocytes are similar. However, little is known about the inducing potency of PCDDs in human cells. Therefore, induction of P450IA1-dependent 7-ethoxyresorufin O-deethylase (EROD) was investigated in the human hepatoma cell line HepG2. The EC_{50}-value for the most potent PCDD, 2378-Cl_4DD (TCDD), determined in HepG2 cells (706 pg/plate) was much higher than those determined in rat H4IIE cells (89 pg/plate) and rat hepatocytes (37 pg/plate). The relative potency of other PCDDs was calculated from their respective EC_{50} values and is given as TCDD equivalents (TE). A similar rank order of TE for the 2378-substituted PCDDs was obtained in human and rat cells, though in HepG2 cells 12378-Cl_5DD (TE 0.75) and 123478-Cl_6DD (TE 0.75) were nearly as potent as 2378-Cl_4DD. Furthermore, Cl_8DD did not lead to detectable EROD induction in the human cell line (TE < 0.001). For a complex PCDD mixture containing 49 congeners the experimentally obtained EC_{50}-value was in good agreement with that calculated from the sum of its 2378-substituted PCDDs, suggesting additive effects of the 6 most potent PCDDs. However, results obtained with mixtures containing > 50% Cl_8DD suggest partial antagonistic effects of Cl_8DD.

K 46

POLYCHLORINATED DIBENZO-p-DIOXINS (PCDDs) AND ETHINYLESTRADIOL AS GROWTH MODULATORS IN RAT HEPATOCYTE PRIMARY CULTURES

A. Karger, D. Schrenk and K.W. Bock
Institute of Toxicology, University of Tübingen, W-7400 Tübingen, FRG

2,3,7,8-Cl_4DD (TCDD) has been shown to act as a liver tumor promotor in female rats (Pitot et al., Cancer Res. 40, 3616-3620, 1980). To elucidate mechanisms responsible for this sex specific tumor promoting activity, the influence of 2,3,7,8-Cl_4DD, 1,2,3,4,6,7,8-Cl_7DD (HCDD) and Cl_8DD (OCDD, containing 0.5% HCDD) on EGF-stimulated DNA synthesis was studied in primary hepatocyte cultures of male and female rats in the presence of 5% fetal calf serum. Addition of TCDD (maximal effect at 10^{-12} M), HCDD (maximal effect at 10^{-11} M), and OCDD (maximal effect at 10^{-9} M) increased DNA synthesis 30-50% in a strictly EGF-dependent manner, the rank order of potency suggesting involvement of the Ah receptor. Induction of P450IA1-dependent 7-ethoxyresorufin O-deethylase activity occurred at PCDD concentrations which were higher than those leading to maximal stimulation of DNA synthesis. Addition of ethinylestradiol further increased TCDD-mediated stimulation of DNA synthesis. This effect was variable. However, the hepatocyte preparations responding strongly to TCDD also responded strongest to ethinylestradiol. In the presence of 3 x 10^{-12} M TCDD a 2.5-fold stimulation of DNA synthesis was obtained at 20 μM ethinylestradiol.
The results indicate that PCDDs enhance EGF-stimulated DNA synthesis in rat hepatocytes in the rank order of their binding affinity to the Ah receptor. Furthermore, synergistic effects of ethinylestradiol suggest that estrogens facilitate tumor promoting actions of PCDDs.

K 47

A NOVEL CONTROL POINT IN CARCINOGENESIS

G. Bauer, P. Höfler, M. Götschl, E. Viesel, J. Jürgensmeier, D. Schaefer, G. Picht
Abteilung Virologie, Institut für Med. Mikrobiologie und Hygiene der Universität Freiburg

TGF-beta plays a dualistic role in the carcinogenesis of fibroblasts. It is both involved in the establishment of the transformed state and in its maintenance via an autocrinous loop. The transforming effect of TGF-beta is due to qualitative changes of the cells and not due to a positive selection process.
This procarcinogenic effect of TGF-beta is directly opposed by a subsequent direct and very potent interaction against transformed cells. This inhibitory effect requires the presence of normal cells and is dependent on the dose of TGF-beta. It results in an effective elimination of the transformed cells due to a cytotoxic effect. Thus there exists a negative regulatory effect of TGF-beta in the process of carcinogenesis beyond initiation and promotion. There are several theoretical possibilities to overcome this inhibitory effect: i) interfering substances, which would contribute to carcinogenesis without necessarily beeing initiators or tumor promoters themselves; ii) resistance of transformed cells , and iii) inability of normal cells to perform TGF-beta-mediated inhibition. First experiments show the existence of interfering substances and demonstrate resistance of certain tumor cells. Further work on this control point might give us more information on cocarcinogenesis and tumor cell progression.

K 48

ROLE OF SOLUBLE FACTORS IN THE TGF-BETA MEDIATED CYTOTOXIC EFFECT OF NORMAL CELLS AGAINST TRANSFORMED CELLS

J. Jürgensmeier, D. Schaefer, P. Höfler, J. Kiefer, G. Picht, G. Bauer.
Abteilung Virologie, Inst. für Med. Mikrobiologie und Hygiene der Universität Freiburg

The TGF-beta mediated elimination of transformed cells by normal cells and interference with this process have been further characterized.
The cytotoxic effect is caused by a soluble agent, which is produced by normal cells after treatment with TGF-beta. Cell-to-cell contact is not necessary for this process: When normal and transformed cells are separated by soft agar and treated with TGF-beta, elimination of transformed cells is still possible. Likewise, supernatant of TGF-Beta treated normal cells transfers a soluble factor, which is responsible for a direct cytotoxic effect on transformed cells.
Interference with the here described cytotoxic effect can be obtained experimentally either with catechol or soluble factors, released from resistant tumor cells. The latter result indicates that tumor cells, which can escape TGF-beta mediated cytotoxic effects, release factors, which protect themselves and possibly mediate resistance to neighbouring transformed cells. Interference with the elimination step by exogenous chemicals or soluble factors from cells may be directly involved in the regulation of tumor development.

K 49

ELIMINATION OF TRANSFORMED CELLS BY A TGF-BETA-MEDIATED CYTOTOXIC EFFECT OF NORMAL CELLS

G. Bauer, P. Höfler, E. Viesel,
J. Jürgensmeier, D. Schaefer,
G. Picht
Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene, Universität Freiburg

Coculture of transformed fibroblasts with a surplus of normal cells in the presence of TGF-beta leads to a net loss of cells exhibiting the transformed phenotype. This effect is dependent on the dose of TGF-beta applied, the duration of coculture, and the density of the normal cells. Several possible alternative mechanims might equally explain the phenomeneon observed: reversible or irreversible inhibition of proliferation, reversion to the normal phenotype, or elimination of transformed cells. Using transformed cells with a neomycin resistance gene or a specific marker, detectable by immunofluorescence, the destiny of transformed cells in TGF-beta-controlled inhibition experiments was followed. Our analysis shows that a massive cytotoxic effect leads to the elimination of most of the transformed cells in coculture. The effect is specifically directed against transformed cells and therefore is discussed as a potent controlling step in carcinogenesis.

K 50

Expression and effects of TGF-ß1 during multistage carcinogenesis in mouse skin

P. Krieg*, R. Schnapke, S. Feil*, G. Fürstenberger and F. Marks, Institute for Virus Research* and Institute for Biochemistry, German Cancer Research Center, D-6900 Heidelberg, Germany

The role of transforming growth factor ß1 in multistage carcinogenesis in mouse skin was assessed by studying its growth inhibitory effects on non-tumorigenic versus tumorigenic keratinocytes and by examining its mRNA expression in vitro and during epidermal hyperproliferation and multistage carcinogenesis. While primary basal keratinocytes have been found to be fully responsive to TGF-ß1 immortal non-malignant cell lines and some tumorigenic cells had partially or completely lost this response to the growth-inhibitory activity of TGF-ß1. Steady state levels of TGF-ß1 mRNA were shown to be high in all tumorigenic cell lines but low in primary basal cells and in non-tumorigenic keratinocyte lines. Furthermore, Ca^{2+}-dependent modulation of TGF-ß1 expression seemed to be lost upon immortalization. Our in vivo studies showed that only tumor promoters, but not mitogenic or weak hyperplasiogenic agents were able to induce a transient expression of TGF-ß1 mRNA in mouse epidermis. A constitutive overexpression of TGF-ß1 mRNA was observed in malignant carcinomas but not in the benign premalignant lesions, indicating that overexpression might be associated with malignant progression. Being interested in elucidating the function and mechanism of this tumor-specific overexpression we will describe the TGF-ß induced modulation of cellular gene expression in malignant versus non-malignant mouse keratinocytes.

K 51

Studies on the inheritance, structure and expression of a xiphophorine gene related to the human gene coding for the PDGF-receptor

E.Wagner, U.Schleenbecker, A.Anders, Ch.Zechel

Two different types of the plateled derived growth factor receptor (PDGF-Rα and PDGF-R ß) are coded by two different human genes and are activated by binding of PDGF homodimers and/or heterodimers. It is supposed that PDGF-Rα and ß are involved in a signal transduction pathway important for the regulation of cell-proliferation in normal cells and in tumor cells.

We investigated inheritance, structure, and expression of the putative pdgf-r gene of Xiphophorus. It appeared that only one type of pdgf-r gene exists in Xiphophorus. We cloned genomic sequences of Xiphophorus that probably encode the juxtamembrane domain, half of the tyrosine kinase domain (TK 1) and parts of the spacer of the putative xiphophorine PDGF-R. Computer mediated sequence analysis revealed that the most striking homology exists between the putative x-pdgf-r sequences and the human gene encoding the PDGF-Rα.

Hybridization of a x-pdgf-r specific probe against mRNA from brain led to the identification of a 5.9 kb mRNA. Besides the brain, eye, gill, heart, kidney, spleen, and testes express the 5.9 kb mRNA. Expression was not detectable in liver and muscle. The 5.9 kb mRNA and a second mRNA of 4.1 kb is stored in the unfertilized egg. In embryogenesis, after decreasing during the formation of blastula and gastrula, the amount of the 5.9 kb mRNA increaes slightly and then stays almost constant during birth and in young fish. In contrast, the amount of the 4.1 kb mRNA decreases completely during embryogenesis in the course of which it becomes undetectable after organogenesis. A mRNA of 4.1 kb was also identified in a melanoma cell-line, but was not detectable in any other cell line and in any tissue of the adult fish.
Supported by the University of Giessen and DFG.

M 1

Approaches to Molecular Cloning of a Putative Tumor Suppressor Gene Controlling HeLa Cell Tumorigenicity

M.M. Gross, S. Unger and E.J. Stanbridge*
Inst. f. Biochemie, DKFZ, INF 280, D-6900 Heidelberg
*University of California, Irvine, CA 92717 USA

The technique of somatic cell hybridization has established the phenomenon on tumor suppression. The work of Stanbridge et al. has provided evidence that a gene located on normal human chromosome 11 can suppress tumorigenicity in hybrids derived from a fusion between HeLa cells and normal human fibroblasts. The strategy used for cloning this gene took advantage of the fact that Hela as well as the tumorigenic Hela x fibroblast hybrid express a 75 kDa cell surface antigen (= intestinal alkaline phosphatase IAP), which is neither present in the fibroblast parent nor in the non-tumorigenic hybrid. Retroviral insertional mutagenesis has been employed to induce expression of IAP in the non-tumorigenic hybrid. From these experiments, 2 independent cell lines expressing IAP and the tumorigenic phenotype have been obtained. Analysis of these clones revealed a small number of inserted retroviruses, ranging from 3 to 4.
We are in the process of isolating the flanking sequences at the sites of retroviral insertion to determine a common integration site in the 2 different hybrid cell lines that regulates expression of IAP and HeLa tumorigenicity. This is being done by conventional screening of genomic libraries of the cell lines using retroviral specific DNA probes. Another approach is the use of the inverse PCR methodology to directly isolate genomic sequences flanking the retroviral insertion sites.

M 2

Dependence of the oncogenic potential of the c-Ha-ras oncogene on the cytogenetic status of the recipient cell.

Petra Boukamp, Ulrich Pascheberg, and Norbert E. Fusenig; DKFZ, Institut für Biochemie, 6900 Heidelberg.

In the last years a number of models were proposed to explain the multistep process of human carcinogenesis. One step is supposed to be correlated with the activation of proto-oncogenes leading to dominantly acting oncogenes. In order to study the role of the ras-oncogene, found to be activated in several human tumors, we transfected spontaneously immortalized human skin keratinocytes (HaCaT) with the cellular Harvey-ras-oncogene. From 18 analyzed clones (HaCaT-ras) 12 were tumorigenic after subcutaneous injection into nude mice. According to the growth behavior and histology these clones were subdivided into two groups: 6 clones forming benign tumors (cysts) and 6 forming invasively growing carcinomas. Since the cells of both groups expressed similar levels of the oncogene product (p21-val12) malignant (versus benign) tumor growth obviously required additional changes. To study this hypothesis, chromosomes of different HaCaT-ras clones which contained the integrated ras oncogene were isolated via micronuclei-transfer and, after fusion with mouse cells, multiplied and analyzed. Identification of the human chromosomes by differential staining (G 11) and G-banding as well as localization of the integration site of the ras-oncogene in the respective chromosomes by in situ hybridization clearly showed random integration, indicating that the integration site was not responsible for the respective phenotype. Transfer of these chromosomes into HaCaT cells of different passage levels further supported our hypothesis that (i) the ras-oncogene enabled the cells to grow in vivo (to give rise to benign tumors); (ii) however, the genetic status of the recipient cell (early versus later HaCaT passages) was causally related with the level of tumorigenicity. This argues against a dominant action of the oncogene and suggests synergistic or additive effect with other molecular events.

M 3

NEOPLASTIC TRANSFORMATION OF ESTABLISHED RAT 208F OR MOUSE 3T3 FIBROBLASTS BY TRANSFECTION WITH AMPLIFICATION PROMOTING DNA SEQUENCES.

H. Abken[1], U. H. Weidle[2], F. Grummt[3], K. Willecke[1].
[1]Institut für Genetik, Abt. Molekulargenetik, Universität Bonn, Römerstr. 164, D-5300 Bonn 1; [2]Boehringer Mannheim GmbH, Nonnenwaldstr. 2, D-8122 Penzberg; [3]Institut für Biochemie, Universität Würzburg, Röntgenring 11, D-8700 Würzburg

Cloned muNTS1 DNA isolated from the nontranscribed spacer of murine rDNA, as well as a group of mouse DNA elements were shown to increase plasmid DNA amplification in mouse cells when linked to selectable marker DNA driven by a truncated promoter (1,2). Here, we have ligated these DNA elements (muNTS-1; element 1, 2, 4, 5, 8, 12) to phyg DNA coding for resistance to hygromycin B driven by a constitutive promoter. After transfection into established rat fibroblasts (208F), DNA of transfected mouse elements persists in low copy numbers (1 - 10 copies per cell) integrated into high molecular weight DNA. Furthermore, expression of a neoplastically transformed phenotype in 5% - 70% of hygromycin resistant colonies was found (dependent on mouse DNA sequence transfected). Transformed colonies induced tumors after injection into NMRI nu/nu mice. Mouse NIH 3T3 cells are also neoplastically transformed after transfection of these mouse DNA elements. In control experiments, transfection of phyg DNA did not induce neoplastic transformation of rat 208F cells or mouse 3T3 cells. The transforming activity of mouse DNA element 4 could be consecutively transferred in 208F cells using total cellular DNA from primary transfectants. After transfection into primary rat fibroblasts, mouse DNA element 4 does not exhibit neoplastic transformation, nor does this DNA element have the capability to immortalize pre-senescent rat fibroblasts. These mouse DNA elements were suggested (1, 2) to stimulate DNA replication directly by providing start sites for DNA replication or by acting as enhancers of replication. Recently, muNTS1 DNA was shown to interact with HMG-I protein (2) that possibly participates in DNA replication or recombination, and with two other proteins not yet identified (3). We speculate that these DNA binding proteins may mediate the activity to induce neoplastic transformation of 208F cells by deregulation of DNA replication.

1. Holst et al., Cell **52**, 355 - 365 (1988)
2. Wegner et al., Nucl. Acid Res. **17**, 9909 - 9932 (1989)
3. Wegner et al., J. Biol. Chem. **265**, 13925 - 13932 (1990)

M 4

HUMAN NTS-1 TUMOR SUPPRESSOR: NUCLEOTIDE SEQUENCE AND FUNCTIONAL ANALYSIS BY SITE-DIRECTED MUTAGENESIS

R. Schäfer, A. Hajnal, J. Balmer, R. Klemenz
Division of Cancer Research, Institute of Pathology, University of Zürich, CH-8091 Zürich

Previously we have molecularly cloned the candidate human NTS-1 tumor suppressor on an 18 kb DNA restriction fragment originating from a revertant cell line. Transfer of NTS-1 sequences into H-ras transformed rat FE-8 cells results in suppression of anchorage independence and a reduction of tumorigenicity. Ras p21 expression is unimpaired in revertant FE-8 cells (1, 2). A genomic subclone harboring 2.5 kb of human NTS-1 DNA is sufficient to induce reversion of FE-8 cells. We have determined the nucleotide sequence of human NTS-1 DNA. Based on the genomic sequence, primers were synthesized and used to isolate NTS-1-specific cDNAs from mRNA of FE-8 revertant cells by reverse transcription and PCR amplification. Three open reading frames encoding possible peptides of 13 to 56 amino acids were identified. The start codons of each of the predicted peptides were modified by site-directed mutagenesis (ATG→TTG mutation). The functional activity of each DNA construct was assayed by transfection into FE-8 cells. All mutated DNA sequences were able to confer anchorage-dependence on H-ras transformed FE-8 cells. We conclude that none of the predicted peptides is the product of the NTS-1 suppressor. Suppression of the neoplastic phenotype in FE-8 cells is probably caused by a different mechanism.

1 Schäfer et al. (1988) Proc. Natl. Acad. Sci. USA 85: 1590-94
2 Cavenee et al., Eds. (1989) Recessive Oncogenes and Tumor Suppression, Cold Spring Harbor Laboratory.

M 5

FIRST DEMONSTRATION OF P53 AND GsPROTEIN (GSP) GENE MUTATIONS IN HUMAN THYROID CELL-LINES (FTC 133/236/238)

P.E.Goretzki, H.Reishaus, M.Demeure, H.Haubruck, J.Lyons, H.D.Röher; Chir.Univ.Klinik Düsseldorf

Only little is known about the genetic changes leading to thyroid cancer. Thus mutational activation of ras oncogenes has been demonstrated in benign and malignant thyroid tumours and a specific tyrosine kinase gene activation (PTC) has been found in papillary thyroid cancer. After establishing 3 permanent cell lines from a low differentiated thyroid cancer of one patient we now investigated the underlying genetic changes in the primary tumour and the consecutive cell lines. From 1986 on the primary tumour demonstrated a p53 mutation at codon 273, which could not be found in tissue from the first operation of 1981. The cell-lines deriving from tissue, resected in 1987 & 1989, additionallly lacked a normal p53 allel. Only in 1986 some tumor tissue demonstrated N-Ras oncogene activation (codon 13) and the cells from 1989 were positive for mutational activation of Gs-protein (codon 201). The established 3 human thyrocyte cell lines will be of significant importance for further investigations on the interaction of p53, ras and GSP mutations in human thyrocytes.

M 6

The tyrosine phosphorylation of v-fms oncogene product.

Sylvia Trouliaris[1], Angelika Hadwiger-Fangmeier[1], Elke Simon[1], Heiner Niemann[2] and Teruko Tamura[1].

1. Institut für Virologie, Justus-Liebig-Universität Giessen, Frankfurter Str. 107, D-6300 Giessen, 2. Institut für Mikrobiologie, Bundesforschungsanstalt für Viruskrankheiten der Tiere, D-7400 Tübingen

The v-fms oncogene of the McDonough strain of feline sarcoma virus (SM-FeSV) encodes a receptor for the colony stimulating factor 1 of macrophages (CSF-1) which exhibits tyrosine kinase activity. The v-fms-product is phosphorylated at tyrosine residues by autophosphorylation or cross-phosphorylation. The cellular counterpart, the c-fms product (receptor for CSF-1),is also phosphorylated at tyrosine residues upon stimulation by CSF-1. In this study, we have compared the tyrosine phosphorylation sites of v-fms and feline c-fms gene products:

1) The tyrosine phosphorylation sites of the v-fms and the feline c-fms product are completely identical.
2) The fms-products are phosphorylated at seven different residues.
3) Four sites (Y-553, Y-696, Y-705 and Y-807) were mapped by tryptic peptide mapping and CNBr-fragmentation.
4) The 4 tyrosine residues were mutated into phenylalanine residues by site-directed mutagenesis to study their role on the transformation potency of the fms-gene product.

M 7

MUTAGENICITY AND CYTOGENETIC EFFECTS (SCE) OF CHLOROPRENE (2-CHLORO-1,3-BUTADIENE)

G. Westphal, Elke Turner, H. Karels, M. Blaszkewicz, H. Peter

Chloroprene is used for the production of plastics (Neoprene™) and glues. The substance has not been classified by the IARC (International Agency for Research on Cancer) in regard to its carcinogenic potential. Since the chemically closely related substance 1,3-butadiene is a proven carcinogen in animal experiments, and the previous data on chloroprene is insufficient and inconsistent, experiments were performed to investigate the mutagenicity of chloroprene.

Chloroprene was purchased from Matthey Co., FRG, as a 50% solution in toluene. Pure chloroprene is unstable. It was therefore freshly distillated for each experiment. Due to the volatile nature of chloroprene, it was preincubated with *S. typhimurium* TA100 in gas tight screw cap vials with or without S9 Mix or glutathione.

A differentiation between cytotoxicity and mutagenicity was investigated in two parallel experiments: for the mutagenicity test the preincubated *S. typhimurium* were processed according to Ames. For the cytotoxicity test an aliquot of the stock culture was diluted $1:10^6$ with PBS buffer, preincubated with chloroprene in the manner described above, and subsequently plated on nutrient agar. This parallel procedure minimized the error due to dilution in the cytotoxicity test.

Reproducible results in terms of mutated revertants could only be obtained when the time interval between distillation and testing was maintained. The time dependent aging of chloroprene was controlled by gas chromatography. The mutagenicity of freshly prepared chloroprene was not enhanced after addition of S9 mix. Addition of glutathione (with or without S9 mix) reduced mutagenicity and cytogenicity of the substance. The mutagenic effect observed was found to increase with the age of the distilled substance.

In additional experiments with human lymphocytes the rate of sister chromatid exchange (SCE) induced by incubation with chloroprene was determined according to the method described by Perry & Wolf. In contrast to literature, an increase of SCE was found. A strong lymphotoxicity was observed as well. The recently described immunosupressive effect of chloroprene could be connected with this toxic effect on lymphocytes.

The results show that aging of glues containing chloroprene could lead to an increase of health risk for the user.

Inst. f. Arbeitsphys., Ardeystr. 67, 4600 Dortmund 1, FRG

M 8

DISLOCATION OF CHROMATIN ELEMENTS IN PROPHASE INDUCED BY 3,3'-DIETHYLSTILBESTROL: A NOVEL MECHANISM BY WHICH MICRONUCLEI CAN ARISE

Helga Stopper, Dietmar Schiffmann and Umberto De Boni*, Inst. of Toxicology, University of Würzburg, F.R.G., * Dept. of Physiology, University of Toronto, Canada.

The mechanisms of estrogen-mediated carcinogenesis are currently under much debate. The synthetic estrogen diethylstilbestrol (DES) transforms Syrian hamster embryo (SHE) fibroblasts neoplastically in vitro without detectable DNA damage. Instead, it is known that DES induces micronuclei (MN) and near-diploid aneuploidy in these cells. In order to clarify the origin of these MN and their possible role as an intermediate step in aneuploidy induction, we have now studied the time course of their formation in living SHE cells during mitosis. The analog 3,3'-DES was chosen to induce MN since it proved to be more efficient than DES. The cells were labelled with DAPI (DNA fluorescence) and monitored live using UV-light and phase contrast microscopy. A S.I.T. (silicon intensifier target) camera was used to allow low UV-dosage in order to avoid cellular damage. Lagging chromosome elements were detected as early as prophase, persisting throughout metaphase/anaphase. These structures formed MN after karyokinesis. Visualization of kinetochores in 3,3'-DES-induced MN by CREST-antibodies yielded a considerable number of CREST-reactive MN (> 60 %) most likely indicating the presence of whole chromosomes/chromatids. Furthermore, these results suggest a causal relationship between the interference of estrogens with the function of the mitotic apparatus, the formation of MN and aneuploidy induction.

M 9

INDUCTION OF MICRONUCLEI BY DIETHYLSTILBESTROL AND ESTRADIOL: COMPARISON OF VARIOUS MAMMALIAN PRIMARY CELLS IN CULTURE

M. Schuler, R. Schnitzler, H. Zankl*, M. Metzler Dept. of Food Chemistry and Environmental Toxicology, and *Dept. of Human Biology and Human Genetics, University of Kaiserslautern, P.O. Box 3049, D-6750 Kaiserslautern, Fed. Rep. Germany

The steroidal estrogen 17β-estradiol (E_2) and the stilbene estrogen diethylstilbestrol (DES) are known to induce micronuclei (MN), aneuploidy and neoplastic cell transformation in Syrian hamster embryo (SHE) fibroblasts. It has been reported recently (Tsutsui et al., Toxicology in Vitro 4, 75-84, 1990) that the aneuploidogenic activities of E_2 and DES are markedly lower in human foreskin fibroblasts than in SHE cells.

We have compared three different human primary cell systems, i.e. amnion fluid cells, chorion villi cells and human peripheral lymphocytes, with SHE and sheep seminal vesicle (SSV) cells with regard to estrogen-induced MN. Significant formation of MN was observed after treatment with E_2 or DES at various concentrations in all human cell systems. In SSV cells, the induction of MN was about the same as in SHE cells. All three human cell types were less sensitive to E_2 and DES treatment than were SHE and SSV cells. These data are in accordance with the results reported by Tsutsui et al. Whether differences in the interactions with the mitotic spindle apparatus or different detoxifying capabilities are responsible for the different susceptibilities of the cells remains to be clarified.

M 10

COVALENT AND NON-COVALENT BINDING OF QUINONES TO MICROTUBULAR PROTEINS: STRUCTURE/ACTIVITY RELATIONS AND IMPLICATIONS FOR MICROTUBULE ASSEMBLY

E. Pfeiffer, R. Aulenbacher and M. Metzler
Dept. of Food Chemistry and Environmental Toxicology, University of Kaiserslautern, P.O. Box 3049, D-6750 Kaiserslautern, Fed. Rep. Germany

For carcinogenic estrogens and benzene, the induction of near-diploid aneuploidy has been proposed as an early genetic event in neoplastic cell transformation. The biochemical mechanisms of aneuploidy induction appear to involve the interaction of quinone metabolites of these agents with the mitotic spindle apparatus. In order to elucidate the aneuploidogenic effects of other quinones and to clarify the structural requirements for this activity, we have studied the interactions with microtubular proteins (MTP) in vitro of several methyl substituted para- and ortho-benzoquinones and naphthoquinones as well as a series of quinones of polycyclic aromatic hydrocarbons, e.g. benzo(a)-pyrene and chrysene. We found that all quinones unsubstituted in the ortho-position and allowing Michael addition are able to bind covalently to MTP and to inhibit microtubule (MT) polymerization. Covalent binding reduced the number of free sulfhydryl groups (determined with Ellman's reagent) compared with control MTP and so involved the MTP cysteines. Moreover, binding to MTP and inhibition of MT assembly could be completely prevented by the addition of cysteine. Interestingly, a marked inhibition of MT polymerization was observed with phenanthrene-9,10-quinone in the absence of covalent binding. Thus, certain quinones of aromatic hydrocarbons are able to interact either covalently or non-covalently with MTP. They disturb MT assembly and may have aneuploidogenic and carcinogenic potential.

M 11

DIFFERENT CYTOGENETIC TOXICITY OF METHYL BROMIDE IN HUMAN "CONJUGATORS" AND "NON-CONJUGATORS"

T. Langhof, K.R. Schröder, H. Peter, E. Hallier

Methyl bromide, which is a direct alkylant, has been classified as a possible carcinogen by the "Deutsche Forschungsgemeinschaft" and the IARC. In 1990, our group reported an enzymatic conjugation of methyl bromide to gluthatione in human erythrocytes. A distinct enzyme polymorphism was observed for this metabolism; 2/3 of the investigated individual blood samples showed a marked enzyme activity (conjugators) whereas 1/3 did not (non-conjugators). The implications of this enzyme polymorphism remained to be clarified.

In the study presented here, individual whole blood samples of 5 conjugators and 5 non-conjugators were incubated for one hour in 22 ml head space vials with 3.6 µmole methyl bromide. Acute cytotoxic effects of the substance on lymphocytes were not observed at the chosen concentration in the samples of either subpopulation.

During the incubation, a rapid disappearance of methyl bromide from the gas phase of the head space vials was observed in the case of the conjugators in contrast to the non-conjugators. Following the incubation, the lymphocytes were isolated from the blood samples, cultivated and processed for SCE-determination according to Perry and Wolff.

The microscopic evaluation showed a substantial increase of SCE in the lymphocytes of each non-conjugator from 6.1±1.65 SCE/mitosis to 11.36±2.45 SCE/mitosis in comparison to samples which had not been incubated with methyl bromide. In the case of the conjugators, no increase of the SCE was observed.

This result indicates a protective effect of the conjugation of methyl bromide to glutathione in erythrocytes which influences the cytogenetic toxicity of the substance to lymphocytes.

Institut für Arbeitsphysiologie an der Universität Dortmund, Abteilung Toxikologie und Arbeitsmedizin, Ardeystr. 67, 4600 Dortmund 1, FRG

M 12

Cytogenetic findings in different types of gastric carcinoma

K. Saal, H.P. Vollmers and H.K. Müller-Hermelink

Inst. f. Pathologie, Universität Würzburg, 8700 Würzburg, Josef-Schneider-Str. 2, FRG

Only a few solid tumors are cytogenetically well investigated, e.g. renal cell carcinoma or lung cancers. The stomach carcinoma is, in spite of its high incidence, cytogenetically only poorly investigated. One of the reasons is the difficulty in culturing the tumor cells. This study describes the chromosome analysis of 27 human gastric cancers from male and female patients in the age of 23-84 years. The tissue was received immediatly after surgery and the cells were mechanically or enzymatically prepared. Mitoses were prepared by direct preparation after 2-17 h or from primary cultures after 1-10 days. Lymphocytes from the same patients served as controls. Karyotyping was done by G- and/or R-banding methods. Significant chromosomal differences could be observed between signet-ring cell carcinomas and adenocarcinomas of the intestinal typ. The relation of tumorigenicity and tumorprogression to the foung aberrations has to be discussed.

M 13

SCREENING OF SIMULTANEOUS MULTIPLE TUMORS IN A YOUNG WOMAN BY CYTOGENETIC AND MOLECULAR METHODS

Henn W., Seitz G., Lagoda P., Christmann A., Blin N., Welter C.
Inst. f. Humangenetik, Univ. d. Saarlandes, Homburg/Saar

In a young female patient (22 y) who had survived an adrenal carcinoma at the age of 2, multiple tumors were detected: a metastatic invasive lobular mammary carcinoma (pT2 GII pNIb), a huge granulosa cell tumor of left ovary (6.8 kg), focal nodular hyperplasias of the liver and uterine leiomyoma. The breast carcinoma and the uterine leiomyoma expressed estrogen receptors but no progesterone receptors. Cytogenetic evaluation of tumors and normal cells (from peripheral blood, healthy liver and fibroblasts) all yielded a normal 46,XX karyotype. Moreover, DNA was isolated from the same sources and tested for DNA fingerprints by a $(GTG)_5$ oligonucleotide and the 33-15 minisatellite. AluI and HaeIII patterns were identical in all cases compared. These results indicate that no gross structural rearrangements of the genome have taken place despite the genesis of (an uncommon) multiple neoplasia.

M 14

Involvement of the tumor modifier locus of *Xiphophorus* in transcriptional regulation of the melanoma oncogene *Xmrk*

D. Adam, D. Förnzler, C. Winkler, W. Mäueler and M. Schartl
MPI-Biochemistry/Gene Center, D-8033 Martinsried

The melanoma inducing gene *Xmrk* of *Xiphophorus* represents a novel receptor tyrosine kinase of the EGF receptor type. We have shown that *Xmrk* can be encoded by three different loci. One locus representing the proto-oncogene is an essential part of the genome and not involved in tumorigenesis. The other two loci were generated by gene duplication/translocation of the proto-oncogene. Both have oncogenic potential after elimination of the tumor modifier locus *R*. Loss of this locus leads to overexpression in melanoma of both oncogenic loci but not of the proto-oncogene. Primer extension analysis revealed the utilization of different promoters in the oncogenic loci compared to the proto-oncogene. A CAT-gene driven by the promoter from one of the oncogenic loci was active in a *Xiphophorus* melanoma cell line but showed only barely detectable activity in an embryonal cell line. In vivo studies in transgenic fish showed differential activitiy of the construct at different embryonic stages. This indicates a transcriptional regulation of the oncogenic loci in fish carrying the tumor modifier locus whereas the proto-oncogene is obviously not regulated by the *R* locus. Analysis of transcriptional control elements acting on the oncogenic *Xmrk* promoters will facilitate the identification and isolation of the tumor modifier locus. In a top-bottom approach methods of reverse genetics were applied to identify the tumor modifier locus. We have defined more precisely the linkage between carboxylesterase *ES1* and the *R* locus. Polymorphic loci with high polymorphism information content have been isolated and tested for their linkage to the *R* locus in order to obtain molecular markers located closer to the *R* locus than the distant isozyme locus.

M 15

EXPRESSION OF THE BREAST CANCER-ASSOCIATED PROTEIN PS2 IN ADENOSQUAMOUS TUMORS OF THE HUMAN GASTRO-INTESTINAL TRACT

Welter C., Theisinger B., Seitz G., Schüder G., Blin N.
Inst. f. Humangenetik, Pathologie und Allgemenine Chirurgie der Universität des Saarlandes, Homburg/Saar

The human pS2 gene, isolated from the breast carcinoma cell line MCF7 and shown to be under estrogen transcriptional control in a subclass of breast cancer cells was reported to be expressed in normal stomach surface epithelial cells whereas additional gastrointestinal tissues like pancreas and colon do not produce pS2 at all. On RNA (Northern blots) and protein (immunohistochemistry) level we have demonstrated pS2 expression in various tumors of the human gastrointestinal tract like carcinoma of the stomach, colon, pancreas and biliary tract. The tumors investigated by us did not show any estrogen receptors neither by immunohistochemistry nor by biochemistry. Thus, the transcription of pS2 is estrogen independent in contrast to breast carcinomas. The mechanism of pS2 activation remains to be elucidated.
The physiological role of the pS2 protein is still unknown. As in breast tumor cells, pS2 immunostaining in stomach mucosa cells was predominantly cytoplasmic with an uneven distribution and often perinuclear localization corresponding to the Golgi apparatus. This result indicates that the pS2 gene codes for a secretory protein. To investigate such function in more detail 14 adenosquamous carcinomas of the human gastrointestinal tract were screened for pS2 expression. Only the adenomatous areas of the tumors were positive whereas the squamous parts remained negative as judged by pS2 staining. Our data support the conclusion that the pS2 protein is being secreted because of its exclusive localization in the adenocarcinomas. First structural evidence suggested that pS2 might be a growth factor or a degrading enzyme of the extracellular matrix.

M 16

Expression of N-myc in human germ cell tumors

Rüther., U., Nunnensiek,C., Müller, H.A.G., Rupp, W., Lüthgens, M., Jipp, P.

Cellular oncogenes are frequently activated of deregulated in human tumor cells. Expression of N-myc was found in significant amounts in neuroblastoma, small cell bronchial carcinoma. Wilms tumor and in human and murine teratoma cell lines. The expression of N-myc in testicular biopsy specimens from 20 patients with testicular cancer seminoma (n=8), embryonal carcinoma (n=5), embryonal carcinoma + teratome (n=1), teratoma (n=4), Leydig cell tumor (n=1) were analyzed by in situ hybridization. Specimens from 7 of 20 patients including all patients with embryonal carcinoma and 1 of 4 patients with teratoma showed intense hybridization for N-myc to numerous but not all tumor cells, whereas the oncogene could not be detected in seminoma of Leydig cell tumor. Our study provides avidence for the association of N-myc activation especially with human embryonal carcinoma. However, the role of N-myc oncogene in tumorigenesis of human testicular cancer needs further investigation.

Present address: Katharinenhospital Stuttgart, Kriegsbergstr.60, 7000 Stuttgart 1, FRG

M 17

ONC-GENE EXPRESSION IN HEMATOPOIETIC TISSUE DURING CHEMICALLY INDUCED MURINE T-CELL LEUKEMOGENESIS

I. Kinzler, M. Gulich, H.J. Seidel
Inst. of Occupational and Social Medicine, University of Ulm

In our laboratory we use a very effective model of T-cell leukemogenesis in adult C57Bl/6 x DBA/2 hybrid mice - a single injection of 50mg/kg of methylnitrosurea (MNU) is followed by a high incidence of thymomas (up to 90% in some experiments) of a variety of T-cell phenotypes (Kreja et. al. Leuk. Res. 9, 321-327, 1985), beginning about 12 weeks after the injection. All thymomas at least showed trisomie 15 (for review see Seidel, Thieme Verlag 1986). Molecular changes at the level of oncogen mutation and oncogen expression have been described for the leukemic cells in this model by several groups. - We were interested to see whether the expression of oncogenes which are known to play a role in the proliferation and/or differentiation of hematopoietic tissues would be altered during the latency period (and also in leukemic cells), as a contribution to the target cell question. N-ras, Ki-ras, myb, myc and sis were studied in their expression in the bone marrow and the thymus after isolation of m-RNA. - We found no detectable Ki-ras expression in untreated animals, but a significant amount as soon as 1 day of MNU application in the thymus and the bone marrow, and also at later intervals during the latency period (and in 5 out of 7 thymonas). Myc expression was absent at day 1 and 3 after MNU, but then reappeared. It was present in all thymomas with increased expression in 2 out of 7. Myb was as in controls during the latency period, in 3 out of 7 thymomas no expression was seen. Sis remained unchanged after MNU application. The presence of mutated genes during the latency period will be studied now.

M 18

MUTATIONAL ACTIVATION OF RAS AND Gs-PROTEIN IN DIFFERENTIATED THYROID CANCER FROM GERMAN AND CALIFORNIAN PATIENTS

P.E.Goretzki, J.Lyons, M.Demeure, O.H.Clark, H.D.Röher, F.McCormick, H.R.Bourne;
Chir.Univ.Klinik Düsseldorf

Mutational activation of Ras oncogenes prove significant importance for a variety of different human tumours. Recently also oncogene activation of genes coding for G-proteins have been demonstrated, with tissue specific stimulation of Gi or Gs. Thus mutational activation of GSP was shown in some pituitary tumours and a single thyroid nodule, while GIP was found in some adrenal tumours. To further investigate the importance of Ras and GSP oncogene activation in human thyroid cancer we assessed 27 German and 10 American tissues from patients with papillary (n=28) or follicular (n=8) thyroid cancer. Tissue sectioning and separate PCR amplification as well as hybridization revealed a heterogeneous distribution for Ras and GSP mutations. While Ras oncogene activation was found only on N-Ras (codon 12/13/61) in German and American tissues with similar frequency (11% vs. 16% of tissue fractions), GSP oncogenes were found more often in German 77/215 (36%) than American 4/51 (8%) tissue fractions ($p<0.001$ by Fisher's exact test). The primary underlying genetic changes in differentiated thyroid cancer from German (low iodide) and American (high iodide) patients is therefore different, which has to be considered, when both patient groups are compared.

M 19

EFFECT OF TOPO II INHIBITORS ON HUMAN MYELOIC CELL DIFFERENTIATION

F. Gieseler, F. Boege, H. Biersack, B. Spohn, M. Clark, K. Wilms
Medizinische Poliklinik, University of Wuerzburg,
D8700 Wuerzburg, FRG

Background: In the course of differentiation, transcriptional regulation of genetic programms are necessary to switch from proliferation to differentiation. The conformational state of the DNA is an important factor for the transcription frequency and is regulated by a group of enzymes called topoisomerases (topos). Several cytostatics such as anthracyclines, epipodophyllotoxines, mitoxantrone, and mAMSA act *via* inhibition of these enzymes.
Results: We have studied the effect of topo II inhibitors on the differentiation of HL-60 cells: mitoxantrone induces granulocytic differentiation, mAMSA inhibits DMSO induced granulocytic differentiation, undifferentiated HL-60 cells are highly sensitive to etoposide and resistant to mAMSA, differentiated HL-60 cells are highly resistant to etoposide and sensitive to mAMSA. We could distinguish different topo II isoactivities with specific pH-activity optima which results in a characteristic pH-activity profile of DNA relaxing enzymes in the nuclear extracts of the cells. Obviously, the isoactivities can be inhibited by different substances shown in a etoposid resistant celline.
Conclusion: Modification of topo II, resulting in the topological alteration of different DNA areas, is a possible molecular mechanism of HL-60 cell differentiation. This can be influenced by the selective inhibition of topo II isoactivities.

Supported by Sander Stiftung, grant 90.0381 and Deutsche Forschungs Gemeinschaft (SFB 172, C9)

M 20

FRACTIONATION OF TOPOISOMERASE II IN A MULTI-DRUG RESISTANT HL-60 CELL LINE REVEALES MULTIPLE ISO-ACTIVITIES WITH DIFFERENT SENSITIVITY PATTERNS.

Fritz Boege, Frank Gieseler, Harald Biersack, Michael Clark and Klaus Wilms
Medizinische Poliklinik der Universität Würzburg, Klinikstraße 6-8, 8700 Würzburg

We have fractionated nuclear extracts from a multi-drug resistant subclone of the human promyelocytic subline HL-60 by anion exchange and chromato-focussing and have isolated at least two structurally and functionally different subtypes of topoisomerase II (Topo II) which are not identical to the known α-and β-iso-forms of the enzyme since both forms are contained in each subtype as monitored by immunoblot analysis. Both subtypes exhibit ATP-dependent pBR322 DNA relaxation and phage P4 DNA unknotting activity. The two subtypes are present in about equal proportion and differ remarkably with respect to pH-optimum and sensitivity to m-amsacrine and orthovanadate. Both types are highly insensitive to etoposide inhibition *in-vitro*, which is in agreement with the >100-fold resistance of the cells to this drug *in vivo*.
Functional and structural alterations of Topo II have been described in certain types of multi-drug resistance of malignant cells. Our findings suggest that both iso-forms of Topo II (α and β) can be subject to epigenetic structural modifications *in vivo*. This would be a candidate mechanism for the regulation of Topo II function, which allows malignant cells to swiftly acquire target protein resistance to Topo II inhibitors such as anthracyclines, epipodophyllotoxines and m-amsacrine.

Supported by Wilhelm Sander-Stiftung, grant 90.038.1, and Deutsche Forschungs Gemeinschaft, SFB 172, C9.

M 21

P1/P2 MYC PROMOTER SHIFT IN BURKITT'S LYMPHOMA CELLS IS A POSITION EFFECT OF THE TRANSLOCATION CHROMOSOME

Axel Polack, Lothar Strobl, Regina Feederle, Matthias Schweizer, Dirk Eick, and Georg W. Bornkamm. Institut für Klinische Molekular-biologie und Tumorgenetik der GSF, Marchioninistr. 25, D8000 München 70, Germany.

Preferential usage of the c-myc promoter P1 is a common feature of Burkitt's lymphoma cells in which the expressed c-myc allele is characterized by mutations or rearrangements within and flanking the first exon. To define mechanisms leading to c-myc deregulation, we have studied the expression of a mutated c-myc allele with and without the influence of sequences from the previously described bvr-1 (Burkitt's variants rearranging region 1) and part of the human immunoglobulin κ locus after stable transfer into Burkitt's lymphoma cells. We used for these experiments the mutated c-myc allele from BL60, a Burkitt's lymphoma cell line carrying a t(8;22) translocation with a particularly pronounced usage of the P1 promoter. Studying the expression of various constructs we could show that a mutated c-myc gene without enhancer is only weakly expressed and that a mutated c-myc gene with or without a κ intron enhancer shows a P2/P1 usage characteristic of a normal c-myc gene. A mutated c-myc gene alone or in conjunction with a reconstructed chromosomal breakpoint carrying part of bvr-1, the κ intron enhancer and κ constant region is thus not sufficient to cause the characteristic promoter shift in Burkitt's lymphoma cells.

M 22

AMPLIFICATION OF MOUSE MAJOR SATELLITE DNA IN LARGE MARKER CHROMOSOMES OF MULTIDRUG RESISTANT EHRLICH-LETTRÉ ASCITES CELLS AS SHOWN BY NON ISOTOPIC IN SITU HYBRIDIZATION

Kopun, M., *Scherthan, H., and Granzow, C.

Inst. für Zell-und Tumorbiologie, DKFZ Heidelberg and *Inst. für Humangenetik und Anthropologie der Universität Heidelberg

It is known that satellite DNA or other repeated sequences can be amplified during selection for drug resistant cells. Such cells usually display gene amplification implicating cytogenetic characteristics including expanded chromosomal or abnormal banding regions. However, a spontaneously arisen, multidrug resistant in vitro cell strain (IC $_{50}$ of colchicine= $3.6x10^{-7}$ M) of Ehrlich-Lettré ascites cells does not show striking changes of the karyotype compared to the parental chemosensitive cell line (IC $_{50}$ of colchicine=$1.8x10^{-8}$ M). The resistant cells were further induced to higher resistance levels by stepwise treatment with colchicine and became capable of growing permanently in the presence of $4x10^{-6}$M colchicine.
The cytogenetic analysis of this stable, highly resistant cell line revealed few large marker chromosomes with tandemly repeated C-bands and several dicentric chromosomes. In situ hybridization with biotinylated oligomer DNA derived from the consensus sequence of the mouse major satellite DNA showed positive hybridization to all C-bands. The samples were further probed with CREST serum (anti-centromere antibodies) which labeled exclusively the distal C-bands (active centromeres). NOR-silver staining of the expanded regions on marker chromosomes was negative. After in situ hybridization of the same probe to resistant mouse x man interspecific hybrid cells, only the heterochromatin of mouse chromosomes was labeled confirming the specifity of the oligomere probe.

M 23

Chromosomal Localization of Retroviral Integration Sites in HeLa/Fibroblast Hybrids

P. Janiaud, D. Rueß, S. Unger, B.M. Mechler and M.M. Gross
Inst. f. Biochemie, DKFZ, INF 280, D-6900 Heidelberg

In the human hybrid cell system, a fusion between HeLa cells and normal human fibroblasts, tumorigenicity is suppressed. With prolonged culture conditions, rare tumorigenic segregants arise. They reexpress a cell surface antigen, IAP (intestinal alkaline phosphatase), which is neither present in the fibroblast parent nor in the tumorigenic segregant. By insertional mutagenesis, we have induced reexpression of the tumor marker IAP in the non-tumorigenic hybrid. From these experiments, 2 independent cell lines expressing IAP and displaying the tumorigenic phenotype have been obtained. Southern analysis of these clones revealed a small number of inserted retroviruses, ranging from 3 to 4.
In situ hybridization of these cell clones using retroviral probes indicate the chromosomal location of the retroviral integrations. Furthermore the genomic sequences, flanking the retroviral insertion sites, which have been derived by cloning and inverse PCR (see also abstract M.M. Gross et al.), can be localized by in situ hybridization and Southern analysis.

M 24

MOLECULAR EVENTS IN EXPERIMENTAL OSTEOSARCOMAGENESIS

P. G. Strauss, K. Müller*, and V. Erfle
GSF-Abteilung für Molekulare Zellpathologie, *GSF-Institut für Pathologie, D-8042 Neuherberg

Multiple steps are considered to be necessary in a cell for deploying a full tumor phenotype. Such events may include the activation of oncogenes, inactivation of suppressor genes, or the effect of mobile elements like endogenous retroviruses. We have analyzed alterations of the structure and expression of these genes in radiation-induced murine osteosarcomas.
1.) Endogenous ecotropic retroviruses, which are activated dose dependently upon irradiation early during the latency period, integrated de novo into the cellular genome. Newly acquired proviruses were found in 60% of the tumors and indicate a clonal growth of these cells. 2.) A variable karyotype was found, with excess of extrachromosomal double minutes containig amplified c-myc sequences. The c-myc gene was found amplified in 30% of the tumors. As a consequence of the c-myc amplification the level of c-myc RNA was increased. 3.) The growth suppresor gene p53 was altered in DNA structure or RNA expression in 65% of the tumors. Tumors that showed high expression of p53 RNA did not reach an advanced stage of osteogenic differentiation. The data indicate that p53, in addition to its role in DNA replication, may have a distinct function in the control of osteogenic differentiation.

M 25

NUCLEAR ACCUMULATION OF P53: AN INDICATOR OF CHROMATIN INJURY?

M. Fritsche, C. Haessler, G. Brandner
Abteilung Virologie, Institut für Medizinische Mikrobiologie & Hygiene der Universität, 7800 Freiburg (Germany)

As a matter of fact, in the nuclei of numerous tumor cells the phosphoprotein p53 is found accumulated in a metabolically stabilized form. As a hypothesis, the regulative function of intact p53 for the cell cycle is thought to be imbalanced if p53 is mutated. Here we report an as yet unknown additional feature of p53: We have observed in normal (mouse late embryonic), immortalised (primate kidney , mouse NIH 3T3) and transformed cells (mouse L929) a drastic and long lasting accumulation of nuclear p53 effected by a number of agents known to act onto cellular DNA. From those agents very potent ones are cytostatic drugs used in tumor therapy, e.g. actinomycin D, cisplatinum, doxorubicin, mitomycin, etoposid, 5-fluoruracil, bleomycin, and gamma-irradiation. In the case of doxorubicin, etoposide, mitomycin and cisplatinum, the nuclear accumulation of p53 is accompanied by formation of multinuclear giant cells representing an increased cell mass up to the 30fold amount of untreated cells. Induction of p53 accumulation by these agents shows indepence from de novo transcription. On the other hand, inhibition of translation by cycloheximide prevents p53 accumulation suggesting de novo translation from a hypothetic RNA stock followed by metabolic protein stabilization by unknown mechanisms. The accumulation through stabilization is confirmed by p53 pulse-chase-labelling with ^{35}S-met followed by immunoprecipitation demonstrating long half-life of p53.
The common feature of the above p53 "inducers" is impairment of chromatin function. Therefore, accumulation of p53 at the site of DNA-injury may indicate an as yet unknown function of p53 in repair or destruction of chromatin.
(Supported by DFG, Br 281/13.2).

M 26

CHROMOSOME 1 CHARACTERIZATION IN PRIMARY NEUROBLASTOMAS BY INTERPHASE CYTOGENETICS

H. Christiansen, J. Schestag, N.M. Christiansen, F. Lampert
Univ.-Kinderklinik, Feulgenstr.12, D-6300 Gießen, Germany

In order to eliminate neuroblastomas with progressive potential by screening cytogenetic data with different prognostic meaning have to be known. In 52 primary tumors we could establish the prognostic significance of numerical and structural aberrations of chromosome 1. Metaphase cytogenetics, however, has the disadvantage of being successful in only 20% of cases, and only few metaphase cells can be analyzed per tumor. We, therefore, turned to interphase cytogenetics which allows not only the evaluation of an unrestricted number of nuclei in the individual tumor (about 1000 nuclei are evaluated routinely), but increased the positive results on numerical behavior of chromosome 1 to over 90% of all tumors studied.
The chromosome 1 centromeric probe 1c (pUC1.77) was used to determine numerical changes by in situ hybridization on methanol/acetic acid fixed nuclei prepared from primary neuroblastoma tumors. The three predominant hybridization patterns, i.e. pUC1.77 signals per nucleus, were: 2 (= disomy of chromosome 1), 3 (= trisomy of chromosome 1), 2 and 3 (=disomy and trisomy of chromosome 1 in two different cell clones).
9 out of 14 stage I and II tumors (=64%) showed 3 or 2 and 3 pUC1.77 signals per nucleus, both 2 stage IVs tumors examined had 3 pUC1.77 signals per nucleus, whereas only 1 out of 12 stage IV tumors had 3 pUC1.77 signals per nucleus (=8%). This strong correlation of trisomy or di/trisomy of chromosome 1 with prognostically favorable tumor stages thus would qualify interphase cytogenetics as a useful method to characterize the neuroblastomas which can be detected by screening.
The application of additional probes as 1pt (BG2.8) in double-hybridization protocols delineated also structural aberrations of chromosome 1 as the neuroblastoma-specific 1p-deletion in interphase nuclei. Its predominant occurrence in advanced clinical tumor stages even further emphasizes the value of interphase cytogenetics.

This study was supported by the Deutsche Forschungsgemeinschaft La165/8-1

M 27

FORMATION OF METHYLATION PATTERNS IN TERATOCARCINOMA CELL LINES

Wolfgang A. Schulz, Andreas Hasse, and Helmut Sies
Institut f. Physiologische Chemie I, Heinrich-Heine-Universität, D-4000 Düsseldorf, Germany

The mechanisms that establish normal patterns of DNA-methylation specific for cell types and developmental stages and aberrant ones in tumor cells are not yet known. Because of their high de-novo methylation activity, embryonic cell lines are particular suitable to study these mechanisms. We have investigated the contribution of several parameters, i.e. gene activity, site and pattern of chromosomal integration, copy number, to the formation of de-novo-methylation patterns on plasmids transfected into the murine teratocarcinoma cell line F9.

In individual cell clones, methylation patterns of CAT-reporter gene plasmids bearing different promoters were found to be specific for each construct, suggesting that site of integration, copy number and pattern of integration (head-to head vs head-to-tail) were not decisive factors. Relative methylation did not correlate with promoter strength or with transcriptional activity determined by CAT assays. Thus, a promoterless construct as well as constructs containing the moderately active SV40 early and the HSV tk promoters, respectively, were found essentially unmethylated, whereas the highly active CMVCAT promoter construct was moderately methylated. Plasmids containing the rat AFP promoter or the mouse metallothionein promoter, both transcriptionally inactive, obtained the methylation levels of the respective endogenous promoters, i.e becoming highly methyland and remaining unmethylated, respectively.

These data suggest that promoter sequence determines de-novo-methylation patterns of the complete transfected DNA, but that promoter activity itself is not important for methylation pattern. In contrast to previous assumptions, de-novo-methylation in teratocarcinoma cells does not necessarily result from transcriptional inactivity.

M 28

THE SPREADING OF DNA METHYLATION ACROSS INTEGRATED FOREIGN (ADENOVIRUS TPYE 12) GENOMES IN MAMMALIAN CELLS

G. Orend[1], I. Kuhlmann[2], and W. Doerfler[1]
[1]Institut für Genetik, Universität zu Köln und
[2]Tierforschungsanlage der Universität Konstanz

The establishment of *de novo* generated patterns of DNA methylation is characterized by the gradual spreading of DNA methylation. We have used integrated adenovirus type 12 (Ad12) genomes in hamster tumor cells as model system to study the mechanism of *de novo* DNA methylation. Ad12 induces tumors in neonate hamsters, and the viral DNA is integrated into the hamster genome. The integrated Ad12 DNA in the tumor cells is weakly methylated at the 5'-CCGG-3' sequences. Upon explantation of the tumor cells into culture, DNA methylation gradually spreads across the integrated viral genomes with increasing passages of cells in culture. Methylation is reproducibly initiated between 30 and 50 map units on the integrated viral genome and progresses from there in either direction on the genome. Eventually, the genome is strongly methylated, except for the terminal 2-5% on either end which remain hypomethylated. Similar observations have been made in tumor cell lines with different sites of Ad12 DNA integration. In contrast, the levels of DNA methylation do not change after tumor cell explantation in several segments of hamster cell DNA of the unique or repetitive type. In the integrated foreign DNA nucleotide sequences or structures or chromatin arrangements are presumed to exist that can be preferentially recognized by the system responsible for *de novo* DNA methylation in mammalian cells.

M 29

PATTERNS OF DNA METHYLATION ARE INDISTINGUISHABLE IN DIFFERENT INDIVIDUALS OVER A WIDE RANGE OF HUMAN DNA SEQUENCES

A. Behn-Krappa, I. Hölker, U. Sandaradura de Silva, and W. Doerfler
Institut für Genetik, Universität zu Köln

Patterns of DNA methylation at 5'-CCGG-3' and 5'-GCGC-3' sequences were determined in about 500 kilobase pairs (kbp), corresponding to approximately 0.02 % of the human genome, by using HpaII and HhaI restriction endonucleases, respectively, and randomly selected cosmid clones of human DNA as hybridization probes. Many of these human DNA sequences were of the repetitive type. The DNAs from human lymphocytes, from a mixture of all blood cells or from several established human cell lines (HeLa, KB, 293, or DEV) were included in these analyses. The patterns of DNA methylation were characterized by often completely or partly methylated 5'-CCGG-3' or by partly methylated 5'-GCGC-3' sequences. Even among individuals of different genetic origins (East-Asian or Caucasian), these patterns proved indistinguishable by the method applied. Similar results emerged when the oncogene c-myb or the lamin C gene was used as hybridization probe. The cytokine-dependent stimulation of human lymphocytes did not affect the stability of these patterns. In the same DNA sequences from several human cell lines, much lower levels of DNA methylation were observed. In human HeLa cells the investigated sequences were unmethylated. The human genome apparently exhibits highly cell type-specific patterns of DNA methylation which were often indistinguishable among different individuals even of quite distinct genetic backgrounds.

M 30

THE REVISED CARBOXYL-TERMINAL SEQUENCE OF THE v-fms ONCOGENE PRODUCT: IMPLICATIONS FOR PHOSPHORYLATION BY CDC2 KINASE:

Ute Smola[1], Dagmar Hennig[1], Angelika Hadwiger-Fangmeier[1], Burkhard Schütz[1], Heiner Niemann [2], and Teruko Tamura[1].

1. Institut für Virologie, Justus-Liebig-Universität Giessen, Frankfurter Str. 107, D-6300 Giessen, 2. Institut für Mikrobiologie, Bundesforschungsanstalt für Viruskrankheiten der Tiere, D-7400 Tübingen, Federal Republic of Germany.

The v-fms oncogene product of the McDonough strain of feline sarcoma virus as deduced from the published sequence (Hampe et al., 1984) and its cellular counterpart, c-fms, which encodes the CSF-1 receptor, differ in only 8 point mutations and 11 unrelated amino acid residues of v-fms replacing the 50 C-terminal c-fms specific residues. Resequencing of the v-fms gene indicated the presence of an additional A residue in position 2809 and further demonstrated that the sequence encoding the C-terminus of v-fms corresponded to a region present in the 3'-noncoding region of the c-fms gene. Thus, the newly found C-terminus, Q-R-T-P-P-V-A-R, differed from the published sequence, R-G-P-P-L. Antibody was raised against the synthetic peptide correspoding to this new sequence. This antibody precipitated the v-fms protein derived from the v-fms transformed NRK cells as well as SM-FeSV infected cat fibroblasts. In agreement with the presence of a cdc2 kinase consensus sequence in the newly found sequence, a synthetic peptide carrying the new sequence served as a substrate for cdc2-kinase in vitro. Phosphoamino acid analyses of the peptide indicated that the threonine residue was phosphorylated. Furthermore, tryptic peptide mapping of gp140$^{v\text{-}fms}$ derived from SM-FeSV transformed NRK cells revealed that the identical v-fms specific peptide was phosphorylated in vivo. The kinase activity of the v-fms product was enhanced after cdc2 kinase phosphorylation in vitro.

P 2

EXCESS CHROMOSOMES #4, #6 and #7 REDUCE THE LATENCY PERIOD UNTIL FORMATION OF THE RAT HEPATOMA CL 52

Kerler R. and Rabes H.M., Institute of Pathology, University of Munich, Thalkirchner Str. 36, FRG-8000 Munich 2

A single clone in rat liver culture CL 52, initiated in vivo by DEN and established in vitro at a late preneo-/early neoplastic stage, progressed to slow growth after 8 months and was finally separated into parallel lines to circumvent loss of this early neoplastic culture (Kerler and Rabes, J.Cancer Res. Clin.Oncol. 114,113-123,1988). 7 karyotypes of the 4 lines at early and progressed stages have already been shown (ibid 115,S26,1989). In summary: analysis at early stages revealed 42,XY,var(1), followed by p-losses on #3 and #11 and lateron chromosome gains in one of the lines (52b). At that time in vivo karyotypes were not yet available, due to a long latency period until tumor formation. Cell lines inoculated into newborn rats at the var(1) stage produced tumors after 23-34 weeks and represented, compared to their appropriate cell lines at a similar time, the additional aberrations +4, t(del(6);?) or +6 and +7. This partially trisomic "tumor" karyotype was reached by some of the cell lines after an in vitro extended progression period of ≈ 6 months. Retransplantation of the tumor, as well as implantation of cell line 52a2, which attained the characteristic trisomy at first, gave rise to tumors within 5½-7 weeks only. The results imply, that excess chromosomes #4, #6 or #7 are advantageous for the growth of this hepatoma. An influence of the once typical der(1)-markers or the now often translocated 3q and 11q cannot be ruled out. Comparison (in prep.)of our results with 27 karyotypes analysed in 17 liver tumors described in the literature, reveal that actually no liver- or carcinogen-specific chromosome change is to be found in these experimental tumors. Gene expression and localization in tumor karyotypes with only 1 or 2 aberrations may finally give more information. However, a rat gene map hardly exists and most genes have only been attached to a specific chromosome, but localization to a detailled band or region remains unclear.

P 1

Determination of Tissue Levels of Monohydroxylated Fatty Acids (HODEs and HETEs) in Normal, Hyperplastic and Neoplastic Mouse Epidermis by Gas Chromatography-Mass Spectrometry and Stable Isotope Dilution

M.Stephan*, W.D.Lehmann*, T.Jacobi#, G.Fürstenberger#
Central Spectroscopy Department* and Institute of Biochemistry#
German Cancer Research Center, D-6900 HEIDELBERG

ABSTRACT

In vitro incubation of mouse epidermis homogenate with arachidonic acid shows the production of small amounts of 8-HETE and 12-HETE by 8- and 12-lipoxygenase, respectively. To investigate the tissue levels of monohydroxylated products of linoleic acid (18:2) and arachidonic acid (20:4) in normal, hyperplastic and neoplastic mouse epidermis an assay was developed based on homogenisation at low temperature, solid-phase extraction, derivatisation and gas chromatography-mass spectrometry (GC-MS). This assay enables the determination of tissue levels of linoleic acid and arachidonic acid down to about 200 ppb and those of the corresponding lipoxygenase products (9- and 13-hydroxyoctadecadienoic acid, HODEs, and 5-, 8-, 9-, 11, 12- and 15-hydroxyeicosatetraenoic acids, HETEs) down to about 3 ppb in samples of about 200 mg of tissue. Quantification is performed by stable isotope dilution relative to [carboxyl-$^{18}O_2$]-labelled analogues added as internal standard to the tissue homogenate.

Determinations were performed in epidermis of adult (7w) mice, as well as in DMBA/TPA papillomas and DMBA/TPA carcinomas. The Table summarizing these data shows markedly elevated tissue levels of 8-HETE and 12-HETE in the papillomas and carcinomas, 12-HETE being the most abundant species.

.....Tissue.....	.....Tissue Level.....	
	8-HETE (ng/g)	*12-HETE (ng/g)*
control epidermis (7w)	71 ± 34 (n=3)	43 ± 28 (n=3)
DMBA/TPA papilloma	672 ± 133 (n=3)	1.898 ± 588 (n=3)
DMBA/TPA carcinoma	134 ± 85 (n=7)	1.775 ± 545 (n=7)

The observed alterations in the tissue levels of lipoxygenase derived products indicate a possible mediator function of HETEs in tumor promotion by TPA and they show that 8- and 12-lipoxygenase reactions provide an important contribution to arachidonic acid metabolism in the papillomas and carcinomas investigated.

P 3

IN VITRO AMPLIFICATION OF BCR/ABL mRNA AS A DIAGNOSTIC TOOL FOR Ph+ CHRONIC MYELOGENOUS LEUKEMIA (CML) AND FOR THE DETECTION OF RESIDUAL LEUKEMIC CELLS AFTER ALLOGENIC BONE MARROW TRANSPLANTATION

G. Dölken, A.A. Fauser, R. Herkert, U. Ragoczy, R. Fritzen, W. Lange, J. Finke, B. Nowicki, E. Schalipp, W. Siegert*, R. Mertelsmann
Abt. Hämatol. Onkol., Med. Univ. Klinik Freiburg und Univ. Klinikum Charlottenburg, Berlin(*)

The Philadelphia chromosome (Ph) is found in about 95% of patients with chronic myelogenous leukemia (CML). The basic molecular event is a translocation of a part of the ABL gene from chromosome 9 into the BCR gene located on chromosome 22. We have established a two-step polymerase chain reaction (PCR) to amplify BCR/ABL mRNA from total RNA prepared from peripheral blood lymphocytes or bone marrow cells from patients with CML. Until now, 57 patients with CML were analyzed by PCR: 45 patients (79%) expressed the K-28 type of BCR/ABL mRNA, 10 (18%) the L-6 type and 2 (3%) both mRNAs. 14 patients with other myeloproliferative syndromes (polycythemia vera, idiopathic myelofibrosis, thrombocythemia) and 20 healthy volunteers were negative for BCR/ABL mRNA by two-step PCR. Because of its high sensitivity this technique is very suitable to follow up patients who received an allogoneic bone marrow transplant for CML. A total of 23 patients were studied up to seven times during intervals of 1-40 months after transplantation. 17 patients were transplanted in chronic phase: they all are alive and in complete clinical and cytogentic remission. 14 are negative by one step PCR, 7 also by two-step PCR. From these data it can be concluded that in most of these patients a clinical and cytogenetic remission is followed - after months or even years - by a disappearance of BCR/ABL mRNA positive cells, i.e. a molecular remission.

P 4

INCREASED UPTAKE OF PLASMAPROTEINS BY TUMORS

U. Schilling, H.J. Sinn, W. Maier-Borst, E.A. Friedrich
Deutsches Krebsforschungszentrum, Inst. für Radiologie und Pathophysiologie, Im Neuenheimer Feld 280, D-6900 Heidelberg, FRG

Rat serumalbumin (RSA) was iodinated in two ways: (1) ^{131}Iodine was directly bound to a tyrosin residue of RSA or (2) ^{131}Iodine was bound to a cumulative marker (tyramin-cellobiosis (TCB) or tyramin-deoxysorbitol (TDS)) and this complex was then covalently bound to the protein. The distribution of the labeled RSAs in rats was monitored by a gamma-camera (Phogamma V, Searle/Siemens, FRG). Data were computed by a Gaede medworker NZ. Five minute frames were taken at different times. Regions of interest (ROI) were positioned over the whole body, liver, left hind leg (tumor region) and right hind leg (muscle region). In previous experiments we were able to show that the uptake of plasmaproteins in different tumors of the rat (transplanted intramuscular into the left hind leg) is higher than in normal tissue. If RSA is iodinated with a residualizing marker (TCB or TDS) the uptake of radioactivity in tumors (e.g. ovarian carcinoma of BDIX rats) is up to five times higher compared to directly labeled albumin (^{131}I-RSA). The whole body radioactivity of rats injected with cumulative labeled RSA decreased slower than in rats injected with directly iodinated RSA. Plasma curves show that the biological half-live of ^{131}I-TDS- or ^{131}I-TCB-RSA is only slightly decreased compared to normal RSA. Biopsies of the tumors and fractionation of the tumor cells show an increase of radioactivity in the lysosomal- and golgi-fractions up to 72 hours post injection. In another series of experiments tumor-bearing rats were injected with Aminonaphtaltyrimid-deoxysorbitol-albumin (ANTDS-RSA) which emitts a green fluorescence (≥490nm) when excited with blue light (380-420nm). Kryosections prepared from tumors 24 hours post injection show a bright fluorescence in the tumor cells but not in muscle cells. From these results we conclude that albumin is taken up by the tumors and is metabolized in the tumor cells.

P 5

Quantitative analysis of monoclonal antibody accumulation in human colon carcinoma after *in vivo* and *ex vivo* application

Löhde,E.; Lück,M.; Raude,H.; Schlicker,H.;*Barzen,G.; Kraas,E.
Dep. of Surgery and Nuclearmedicine, Hospital Moabit, Berlin
*Dep. of Nuclearmedicine, University Rudolf-Virchow, Berlin

The crucial point of immunoscintigraphic diagnosis of colo-rectal cancer is the amount of monoclonal antibodies accumulating in the tumor. Aim was to define the mab quantity in the tumor after *in vivo* application in comparison to the scintigraphic detectability pre-operatively and to investigate mab accumulation limits by perfusing the tumor post-operatively in an *ex vivo* perfusion system.

Methods: The mab BW431/26 was i.v. applicated 48h prior to the operation in 10 ptx. suffering from colon cancer. All ptx. gave informed consent.. Immunoscintigraphy was performed 24h prior to the op.. After tm-resection measurements of tissue activity and tm-volume as well as immunochemical work up were done. Additionally 5 of these tumors underwent a further mab challenge by perfusing the resected colon-segment in an oxigenized *ex vio* perfusion system (meth.:Löhde,E; Br.J. Cancer 62, suppl.X 1990) using the same mab in higher concentrations.

Results: The scintigraphic tumor localization pre-op. was positive in 6 cases, 1x indefinite and 3x neg.. However all tumors turned out to be marked by the mab. The range was 0.1-1.8ug /g respectively 0.01-0.09% of the i.v. given mab. The *in vivo* detectability turned out to require a total uptake of 5-6ug mab in the tumor being the product of tm-volume and specific accumu-lation per g tissue. The *ex vivo* perfusion can increase the mab uptake but 3.5ug /g seems to be kind of limit. Immunochemnistry shows heterotopic mab localization.

Conclusion: The data quantify mab enrichment in human colon cancer and permit calculations for tumor targeting.

P 6

A MOUSE MODEL FOR THE GVH-DISEASE (GVHD)-ASSOCIATED GRAFT-VERSUS-LEUKEMIA (GVL) EFFECT

J. Milleck
Zentralinstitut für Krebsforschung Berlin-Buch

GVHD is induced by intravenous injection of parental spleen cells into sublethally irradiated F1 hybrids of the mouse strains XVII/Bln (H-2k) and C57Bl6/Jena (H-2b). Dependent on the number of injected spleen cells F1 hybrids develop a lethal GVHD.
To estimate the GVL effect the GVH-diseased mice are challenged with 10^3 cells of a virus-induced,aggressive myeloblastic leukemia which had been established in mice of the strain XVII/Bln. Seven days later spleens of mice are examined for vital leukemia cells by injection of spleen cells into naive mice of the strain XVII/Bln. If spleens contain vital leukemia cells recipient mice will die during 4 weeks. Results of 5 experiments show that 27 % of the GVH-diseased mice had been protected from the growth of leukemia cells. In this case the GVL effect had been achieved although leukemia cells and donor lymphocytes did not differ genetically.

P 7

Lymphocytic infiltration in renal cell carcinomas and oncocytomas *

R. Keymer[1]; S. Störkel[1]; T. Reichert[1]; F. Steinbach[2]; R. Lippold[3] and W. Thoenes[1]
Institut für Pathologie[1], Urologische Klinik[2] und IMSD[3] der Universität Mainz

The analysis of tumour cell characteristics and lymphocytic infiltration enables to study the mechanisms of tumour defense. We investigated 60 epithelial renal cell tumours with respect to MHC- and integrin-expression and compared the results with quantitative and qualitative findings of lymphocyte subsets (B-, T-, LFA-1$^+$-, CD4$^+$-, CD8$^+$-, CD16$^+$-cells). We further focussed on the topography of the lymphocytic infiltration and on the different tumour types. The infiltration density correlates with ICAM-1- and MHC-I- expression on the tumour cell membrane. ICAM-1 positive clear cell carcinomas and the majority of chromophilic carcinomas show a higher infiltration rate than ICAM-1 negative oncocytomas, chromophobic carcinomas and some ICAM-1-negative chromophilic carcinomas. The infiltration density depends as well on the differentiation of the tumours (cell type, grading) as on some lymphocyte subsets. T-helper cells and B-cells accumulate in the tumour surrounding tissue whereas cytotoxic T cells and NK cells are mainly situated in peripherial tumour areas. The results obtained can be the basis for further investigations to study the effect of immunologic response modifiers.

(*supported by DFG STO 197/1-2)

P 8

Histological factors influencing prognosis in oral squamous cell carcinomas: a multivariate analysis

T. Reichert[1], S. Störkel[1], R. Keymer[1], R. Lippold[2] and W. Wagner[3] Dept. of Pathology[1], Medical Statistics[2] and Maxillofacial Surgery[3], University of Mainz, FRG

Staging and grading are the basic parameters to predict the clinical outcome of patients suffering from carcinomas. We studied retrospectively 100 cases of surgically removed oral squamous cell carcinomas (follow up > 5 years) and compared the prognostic value of the following parameters: Malignancy grading according to Broders (1920), tumor front grading according to Anneroth (1987), degree of keratinization, nuclear atypia, the number of mitotic figures, pattern of invasion according to Jakobsson (1973), and the number of tumoral und peritumoral tissue infiltrating T-, B-lymphocytes and marcophages. The prognostic value of all parameters and different scores was proven statistically with Cox multivariate survival regression analyses.
Results: The tumor front grading is a better prognostic indicator than Broders' grading (CHI-SQARE 17.1/12.5, P= 0.0000/0.0004). Keratinization, nuclear atypia, and number of mitotic figures indipendently have a significant effect on the prognosis (CHI-SQARE 6.5/14.7/17.6, P= 0.0108/0.0001/0.0000). The pattern of invasion is an important prognostic indicator (CHI-SQARE 21.8, P=0.0000): The worse the borderline is defined, the worse is the prognosis of the patient. T-lymphocytes are the predominant infiltrating cell type in the tumor and in the peritumoral tissue, whereas B-lymphocytes are found nearly absent. Only the T-lymphocytes in the peritumoral tissue are of prognostic value (CHI-SQARE 13.6, P=0.0003). The marcophage infiltration exhibits no correlation to the prognosis. The statistical analyses favours the malignancy score with following parameters: Degree of keratinization, nuclear atypia, number of mitosis, pattern of the invasion front and degree of peritumoral T-lymphocyte infiltration (CHI-SQARE 26.1, p=0.0000). This score resulted in a superior prediction of the survival rate.

P 9

PCNA and EGF-R characteristics of oral squamous cell carcinomas

S. Störkel[1], T. Reichert[1]. K.-A. Reiffen[2], and W. Wagner[3] Dept. of Pathology[1] and of Maxillofacial Surgery[3], University of Mainz, FRG; Fa. Merck[2], Darmstadt

Growth factors are known to induce DNA replication and cell division in a variety of human carcinomas. We investigated 100 squamous cell carcinomas of the oral cavity with respect to histological grading, membraneous staining for the EGF-receptor, nuclear PCNA (proliferating cell nuclear antigen) - expression and mitosis count. The immunohistological results were combined in a score (product of the number of positive stained cells and the staining intensity). Using a histological tumor front grading which is superior to the Broders'grading (Bryne et al., 1989) there were 36 carcinomas of grade 1, 32 carcinomas of grade 2 and 32 carcinomas of grade 3. The membraneous EGF-R expression was always accentuated at the tumor front with a mean staining score of 8.5 for grade 1-, 11.5 for grade 2- and 13.0 for grade 3 carcinomas (correlation coeffizient of the linear regression r=0.642). The PCNA preparations showed a qualitative wide range of positive nuclei with a medium staining score of 1.7 for grade 1-, 2.4 for grade 2- and 3.1 for grade 3 carcinomas. These PCNA-data were in good correlation to the real mitosis count (r=0.733). Our results point out:
1. The PCNA-staining score shows a good correlation to the real mitosis count, which proves the PCNA antibody as a valuable tool to detect proliferating cells in paraffin sections;
2. There is a close relationship between the tumor front grading on one hand and the expression of the EGF-receptor and the proliferation rate on the other hand. Thus enhanced expression of the EGF receptor is closely related to tumor proliferation and finally to the prognosis in sqamous cell carcinomas.

P 10

ENDOGENOUS LECTINS: A MARKER CLASS IN NEUROPATHOLOGY ?

A. Bardosi, D. Brkovic, H-J. Gabius
Inst. of Pathology, Detmold, Max-Planck Inst. for exp. Medicine, Göttingen.

The carbohydrate part of glycoconjugates (glycoproteins, glycolipids, proteoglycans) appears to fulfill a prerequisite to serve as carrier of biological informations in a biological recognitive system of protein-carbohydrate interactions by the inherent potential for structural diversity. Evidence for regulation and structural diversity can be detected by sugar-specific probes. Using an assortment of labelled synthetic neoglycoproteins and naturally occuring glycoproteins with different sugar moieties, the glycohistochemical patterns of endogenous sugar-binding proteins, called endogenous lectins, were analyzed in tumors of human peripheral (neurinomas and neurofibromas) and central nervous system (meningiomas of different subtypes, differentiated and anaplastic oligodendrogliomas, ependymomas, glioblastomas, gangliocytomas and astrocytomas). The glycohistochemical spectra of endogenous sugar receptors were different in the distinct types of tumors. Differences of the staining patterns could be observed within the same tumor, showing selective staining of structures characteristic for a given tumor type (whorl formations or lobulated arrangement of the tumor cells in meningotheliomatous and submalignant meningiomas). There were differentiation dependent changes in the pattern of endogenous lectins in tumor cells, suggesting the possible involvement of glycoconjugate-endogenous-receptor system in the cell transformation and development. Exemplary biochemical analysis by affinity chromatography of glioblastomas revealed presence of various proteins with specific sugar-binding capacity, supporting the glycohistochemical results. Our glycohistochemical and biochemical observations on endogenous lectins suggest the potential value of this class of proteins to aid in tumor classification and in understanding of mechanism of tumor differentiation and development.

P 11

Quantitative Determination of CA 15-3, Cathepsin D, gamma-Glutamyltransferase, estrogen and progesteron receptor in Breast Cancer Tissue

Brandt B., Jackisch C., Seitzer D., Hillebrand M., Inst. f. Klin. Chem. d. Universität Münster

In tumour tissue of 96 patients suffering from primary breast cancer the tumour marker CA 15-3, Cathepsin D, gamma-Glutamyltransferase, estrogen and progesteron receptor were studied. The preparation of the examinated compartments based on ultracentrifugation methods at 100,000xg, 4°C. The assay results were not dependent on age and menopausal status. A significant reciprocal relation was revealed between histological grading and CA 15-3 concentration in cytosol and membrane fraction. Multifactorial cox analysis and Kaplan-Meier lifetable analysis revealed a significantly increased risk of cancer recurrence with CA 15-3 concentrations in cytosol and membrane fraction below 40 U/mg ($p < 0.01$) and cathepsin D values in cytosol above 35 pmol/mg ($p < 0.05$). Cytosolic values of our parameter panel from patients with hyperplasia (mastopathia I-III), lymphangiosis carcinomatosa and perinodulous carcinosis of fat tissue have been compared with those of patients without this negative factor of prognosis. A relation to elevated specific activities of gamma-glutamyltransferase in cytosol indicated with lymphangiosis carcinomatosis ($p < 0.05$) and perinodulous carcinosis ($p < 0.05$).
The measurement of biochemical parameters in breast cancer tissue improved to be a valid complementation of morphologically qualitative diagnosis, especially considering prognostic criteria at the time of first therapy decision.

P 12

IMMUNOPHENOTYPING OF ROUTINELY PREPARED PERIPHERAL BLOOD AND BONE MARROW SMEARS AFTER PROLONGED STORAGE WITH CLINICAL VALUE IN THE DIAGNOSIS OF MALIGNANT DISEASE

S. Wehner, B. Kornhuber and U. Ebener
Clinic of Pediatrics, Dept. of Hematology and Oncology, J.W. Goethe Univ., Theodor-Stern-Kai 7, D-6000 Frankfurt/M. 70, Germany

Routine haematological blood smears are appropriate to identify cells with phenotypic characteristics in an immunological assay in conjunction with specific monoclonal antibodies (MoAbs).
In the present study we describe an extensive investigation of long-term stability of antigenic reaction derived from normal lymphocytes or from tumour cells. The phenotyping of routinely prepared and unfixed air dried peripheral blood and bone marrow smears after prolonged storage is demonstrated. We preferred the APAAP labeling reaction with monoclonal complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase carried out in smears under different storage conditions. We evaluated more than 100 cases of smears stored either at room temperature or at 4°C, using a large panel of MoAbs reacting with T- and B-cells, myeloid-monocytic cells, activated and proliferating lymphocytes and MoAbs binding at various stages of differentiation. The retrospective analyses were performed at smears of blood and bone marrow samples from normal donors and from patients with haematological disorders including acute leukemia (n=30) and neuroblastoma (n=7). The results were compared with the results received from freshly prepared mononuclear cells at time of diagnosis. Depending on conditions such as the quality and density of cells and the size of smears itself on the slide up to six separate reaction fields are available for the typing procedure. The flooding and mixture of applied antibodies between the different reaction fields is excluded by the usage of self developed Silicon-Chamber-System (Hanno-SCS). To facilitate the analysis of the cell morphology the cells were counterstained after Fast-Red-Indicator-Reaction using Hemalaun. Analysing smears we could detect the typical marker profile in all cases of storage up to 19 month at 4°C. Surface markers as well as intracytoplasmic or nuclear antigens could be detected in each case. A loss of antigens under the refrigerated conditions was not observed with the exception of smears stored at room temperature for more than 4 weeks. These findings strongly suggest that it is suitable to send unfixed blood and bone marrow smears to reference laboratories during a period of several weeks.

P 13

ANALYSIS OF HUMAN TUMOR CELL PROLIFERATION BY COMBINATION OF Ki-67 ANTIGEN DECTECTION AND LABELLING WITH ^{3}H-THYMIDINE AND 5-BROMODEOXYURIDINE

F. A. Habermann and H. M. Rabes
Pathologisches Institut der Universität München

The cell cycle-dependent appearance of the proliferation-associated nuclear Ki-67 antigen was analysed on non-synchronized exponentially growing human colon carcinoma cells in vitro. Parallel monolayer cultures of HCT-8 cells were successively pulse-labelled with ^{3}H-thymidine (^{3}H-TdR) and 5-bromodeoxyuridine (BrdU) in variable intervals. Ki-67 antigen, ^{3}H-TdR and BrdU were detected simultaneously by immunocytochemical double staining and autoradiography. By this method it was possible to calculate the duration of the cell cycle and its phases, to determine the cell cycle position of individual cells and to analyse their phase-specific Ki-67 antigen expression microscopically avoiding artefacts due to cell synchronization.
Cell cycle progression was characterized by low nucleolar Ki-67 antigen content in early S phase, accumulating deposits in the entire karyoplasm during late S and G2 phase and highest levels at mitotic chromosomes. After telophase Ki-67 antigen gradually decreased and was redistributed to nucleoli.

To investigate cell proliferation in human colon carcinomas in vivo surgically resected tumor-bearing colon segments were exposed to ^{3}H-TdR by vascular perfusion under simulated physiological conditions. Ki-67 antigen and ^{3}H-TdR were visualized simultaneously on kryostat sections by immunostaining and autoradiography. Growth fraction determined by Ki-67 and S phase compartment could be correlated directly on identical slides. The quantitative difference of Ki-67 antigen expression between G1 and G2M cells was used to identify proliferating tumor cells in G1, S and G2M phase and to study their distribution within the preserved tissue architecture.
The different carcinomas showed a relatively constant distribution of the proliferating tumor cells to the cell cycle compartments G1, S and G2M irrespective of the widely varying size of the growth fraction.

P 14

CHARACTERIZATION OF CHEMICALLY INDUCED TUMOR-ASSOCIATED PROTEIN VARIANTS SYNTHESIZED IN RAT HEPATOMAS AND RAT HEPATOMA CELL LINES

Zeindl-Eberhart, Evelyn, and H.M. Rabes
Institute of Pathology, University of Munich, Thalkirchner Str.36, D-8000 Munich 2, F.R.Germany

Tumor-associated proteins have been determined after separating soluble proteins from N-methyl-N-nitrosourea-induced rat hepatomas and transformed rat liver cell lines by high resolution 2-dimensional gel electrophoresis (2DE). Corrections for variants due to cell proliferation and culture conditions were made by comparison with gels from regenerating liver and short-term cultivated liver cells.
Among these tumor-associated protein variants one spot series was detected consisting of six different variants.Three of these spots were localized at MW 35.000, three others at MW 35.500. All six variants had only minor pI differences (6.6, 6.7, 6.8, 7.0, 7.3 and 7.4). One variant was up-regulated only in hepatomas and transformed cells. Another variant was significantly up-regulated in hepatomas and transformed cells but also slightly expressed in short-term cultivated cells. Both variants may represent tumor-associated proteins. As they were not detectable in controls and regenerating liver, they may be related to carcinogenesis. Four variants showed a reduced expression in tumors, transformed cell lines and short-term cultivated cells, thus being putative organspecific proteins and changed by dedifferentiation processes.
Because of the similar position in 2DE gels these different isoelectric variants may result from a single gene or from two genes which show an altered expression, or gene products which are the result of altered posttranslational modification during carcinogenesis.

P 15

EFFECT OF AGING ON NUMBER AND SIZE DISTRIBUTION OF SPONTANEOUS AND DEN-INDUCED ENZYME-ALTERED FOCI IN RAT LIVER, WITH AND WITHOUT PHENOBARBITAL PROMOTION

C. Robl and H. M.Rabes , Institute of Pathology, University of Munich, Thalkirchner Str. 36, FRG-8000 Munich 2

Age-dependent carcinogen sensitivity of the liver was measured by inducing ATPase-deficient foci in male young adult (6-8 months), old (18-20 months) and senescent (28-34 months) Wistar AF/Han rats by N-nitrosodiethylamine (DEN) (5 mg/kg/day) for 14 days in the drinking water following partial hepatectomy (PH). Untreated and merely hepatectomized rats served as controls. To evaluate the effects of promotion, other groups of rats were treated identically but additionally fed 0.05 % phenobarbital (PB) for 11 weeks, beginning at the 15th day after PH. After 3 months number/cm^2 and size of ATPase-deficient foci were measured. In carcinogen-treated rats a non-significant decrease of foci number is observed with increasing age, whereas control groups show a significant increase of spontaneous foci with age. The mean size of foci increases with age in all groups except the PH-DEN group, where it decreases significantly in senescent rats after a maximum in 18-20 months old animals. When comparing carcinogen-treated groups with controls, the age-related increase of the mean foci size of DEN-treated rats is apparently due to a shift of this mean size to a higher value by a high number of pre-existing foci in old and senescent rats (the mean foci size of which exceeds the mean foci size of DEN-treated animals), and less likely to increased cell proliferation. PB treatment generally increases the number of DEN-induced as well as of spontaneous foci. The promotion effect (calculated as difference of the mean area percentage occupied by foci between PB- treated and their corresponding groups) increases with age in DEN- treated rats and PH- controls. Age-related differences show the same tendencies as in non-promoted groups and are more distinct. It is concluded that DEN sensitivity is less expressed in the liver of old rats, while occurrence and size of spontaneous foci increase with age. The response to promotion appears more pronounced in older animals.

P 16

INITIAL REPAIR OF O^6-ETHYLDEOXYGUANOSINE (O^6-EtdGuo) IN DIFFERENT RAT LIVER CELL LINES AFTER N-ETHYL-N-NITROSOUREA (ENU) EXPOSURE

V. Röttgen and H.M. Rabes
Institute of Pathology, University of Munich,
Thalkirchner Str. 36, D-8000 Munich 2, F.R.G.

The capacity for removal of O^6-EtdGuo was determined in two tumorigenic rat liver cell lines CL 38 and CL 49 and a non-tumorigenic rat liver cell line NT 27 which were established in our laboratory (Kerler and Rabes, 1988). A monoclonal antibody (gift from Dr. M. Rajewsky, Essen) was used to evaluate by an immuno slot blot method (Nehls et al., 1984) with slight modifications the molar concentration of O^6-EtdGuo after ENU exposure. The initial concentration was 0.7, 0.9 and 1.2 fmol O^6-EtdGuo/µg DNA for Cl 38, CL 49 and NT 27, respectively, after the end of a 20 min ENU exposure (100 µg/ml). After 60 min only 8% of the initial value was found in the non-tumorigenic line NT 27, whereas 15% and 20% of the initial value were observed in CL 38 and CL 49, respectively. As the low initial concentration of O^6-EtdGuo in these cell lines is in contrast to the higher O^6-EtdGuo concentration seen in DNA of rat liver 30 min after an intravenous injection of ENU (50 µg/g body weight) we determined the formation kinetics of O^6-EtdGuo in DNA of the cell lines during the 20 min exposure to ENU. 5 min after start of ENU exposure the first O^6-EtdGuo is detectable. Its concentration rises until the end of the ENU exposure at 20 min to its maximum. At this time, however, 25% of heterolytic decomposing ENU is still present in the medium at pH 7.2. We excluded further the possibility of an influence of the rate of proliferation on different loss of O^6-EtdGuo in the cell lines by measuring the elimination kinetics of ^3H in DNA after a ^3H-TdR pulse for 25 min. The half-life of the specific activity of DNA yielded values of 48 h, 36 h and 55 h for CL 38, CL 49 and NT 27, respectively, indicating that the rate of proliferation of the cell lines is too slow to be responsible for the differences of O^6-EtdGuo elimination kinetics. Experiments with other tumorigenic and non-tumorigenic liver cell lines will clarify whether the neoplastic cells possess as a rule a lower initial repair capacity than non-tumorigenic liver cell lines.

P 17

Effects of N-Methyl-N-Nitro-N-Nitrosoguanidine and Diethylnitrosamine on fetal hamster tracheal explants

C. Nowak, H.-B. Richter-Reichhelm
Max-von-Pettenkofer Institut,
Bundesgesundheitsamt Berlin

Tracheas were excised from fetal Syrian golden hamsters on the 15th day of gestation. The tracheal explants were cultivated in vitro and exposed to different dose levels of N-Methyl-N-Nitro-N-Nitrosoguanidine (MNNG) or Diethylnitrosamine (DEN). After a 24h-treatment the nitrosamines were washed off and the organ explants were cultivated for another 28 days. After fixation tracheal explants were transversely cut with serial section techniques and scored for morphological changes of the epithelium by light microscopy.
Most of the control tracheal explants showed normal morphology after 4 weeks of in vitro cultivation, except for some decrease in the number of ciliated cells and minimal squamous metaplasia.
DEN concentrations of 0.1, 1, 10 and 100 ug/ml and MNNG concentrations of 0.1, 0.5, 1, 5 and 50 ug/ml led to various changes of the epithelium, such as squamous metaplasia or hyperplastic foci. But a stringent dose-dependent increase of metaplastic or hyperplastic events could not be determined. Nevertheless this in vitro system seems to be suited to detect preneoplastic changes after nitrosamine treatment.

P 18

STUDIES ON THE PREVALENCE OF RAS GENE POINT MUTATIONS IN N-METHYL-N-NITROSOUREA(MNU)-INDUCED INTESTINAL TUMORS OF THE RAT

V. Waldmann and H.M. Rabes
Institute of Pathology, University of Munich,
Thalkirchner Str.36, D-8000 München 2, F.R.G.

Recent investigations revealed ras gene point mutations to be involved in particular in human intestinal carcinogenesis, but also in a number of experimental carcinogenesis model systems using MNU as initiating carcinogen. However, both time and necessity of activation of ras genes for the carcinogenesis process remains unclear.
Aiming at a sequential analysis of the role of ras gene mutations during intestinal carcinogenesis we established an experimental rat tumor model: Male Wistar AF/Han rats were partially hepatectomized, infused via tail vein from 14 to 24 hours after operation with hydroxyurea (HU) and received a single dose of MNU (25 or 50mg/kg) at the start of DNA synthesis after HU block. This treatment resulted in a high incidence of intestinal adenomas and carcinomas, besides liver tumors.
DNA of the induced adenomas and carcinomas was extracted and amplified by PCR using oligonucleotide primers up- and downstream of codon 12 and 61 of H-, K- or N-ras. In dot blots amplified DNA was evaluated for the presence of point mutations by hybridization with ^{32}P-labelled oligonucleotide probes specific for all possible point mutations at codon 12 or 61. Inspite of the ability to detect even low proportions of in vitro-synthesized test oligonucleotides with mutations at specific sites we did not detect any point mutation of ras genes in 39 tumors.
We conclude that ras gene point mutations are not involved in MNU-induced intestinal carcinogenesis of Wistar rats.

P 19

THE ROLE OF K-RAS POINT MUTATIONS IN PRIMARY AND METASTATIC HUMAN COLON CARCINOMAS. A CORRELATION OF MOLECULAR RESULTS AND CLINICAL DATA.

B. Suchy, Ch. Zietz, and H.M. Rabes.
Institute of Pathology, University of Munich
Thalkirchnerstr. 36, 8000 Munich 2, F.R.G.

It is not known wether ras mutations are involved in early stages of colon tumor formation or correlate with tumor progression. We analysed material from paraffin sections of 110 colon tumor patients. PCR, dot blots and allele-specific analytical oligonucleotides were used to detect point mutations. Mutations in K-ras codon 12, position 2 were found in 21.8 % of these samples. This is a similar frequency as described in the literature for the analysis of fresh material from colorectal carcinomas. Direct comparison of primary tumors and metastases revealed a homologous pattern of either occurrence or absence of K-ras mutations in both groups. Metastases do not show a higher incidence of ras gene mutations than primary tumors. Mutation pattern of primary tumors and metastases are identical with respect to GAT transition and GTT transversion mutations. A comparison of the clinical data from patients with and without K-ras codon12/2 point mutations revealed nearly identical values with respect to age, sex or tumor type. Differences were observed in tumor localization with the prevalence of point mutations being slightly higher in coecum tumors than in sigmoid tumors. Mutations were found exclusively in T3 and T4 tumors. The grade of differentiation was equal in both groups. With regard to ploidy tumors with aneuploid pattern showed a higher prevalence of ras mutations as compared with euploid tumors. A clonal selection of cells with K-ras mutation during the metastatic process is unlikely.

P 20

DEMONSTRATION OF TEMPORAL AND TOPICAL C-MYC EXPRESSION IN REGENERATING RAT LIVER AND IN NEOPLASTIC NODULES BY IN SITU HYBRIDIZATION

M.Sarafoff and H.M.Rabes
Institute of Pathology, University of Munich
Thalkirchner Str. 36, 8000 München 2, FRG

In situ hybridization of tissue sections was used to detect in rat liver the temporal and topical expression of c-myc mRNA known from biochemical studies to be elevated during regeneration and hepatocarcinogenesis. The expression of c-myc was studied 1,3 and 6 hr after partial hepatectomy (PH) and in liver nodules produced by continuous administration of diethylnitrosamine (5 mg/kg/day for 42 days). Frozen liver sections, fixed in 4% buffered paraformaldehyde, were hybridized to ^{35}S-labelled RNA-probes of a rat c-myc, produced from a 240-base pair PCR amplified fragment of exon3, subcloned into a pBS vector. The specificity of the hybridization with the antisense RNA was controlled by using ^{35}S-labelled sense transcripts as a negative control. Expression was found to be correlated with cell proliferation: a significant transient increase of c-myc mRNA was observed between 1 and 3 hr after PH. Six hr after PH the level of c-myc mRNA decreased to a nearly normal value.Despite the wellknown specific intralobular distribution of DNA synthesizing hepatocytes as a function of time after PH, only minor topical differences were found for c-myc expression. A quantitative evaluation is under way. High c-myc levels were observed in neoplastic liver nodules. Liver tissue ajacent to the nodules showed low c-myc expression. The results suggest that expression of c-myc is activated shortly after PH without striking differences in the liver lobule, but reverses to normal values rapidly, in contrast to neoplastic nodules with a constitutively enhanced c-myc expression.

P 21

Mutational activation of the H-, K- and N-*ras* genes is generally not involved in initiation and progression of carcinogen-induced liver tumors of the rat

Richard Ostermayr and Hartmut M. Rabes
Institute of Pathology, University of Munich
Thalkirchnerstrasse 36, 8000 Munich 2

Functionally important sites of the H-, K- and N-*ras* genes were isolated from small areas of histologically characterized cryostat sections by the Polymerase Chain Reaction and analyzed by analytical oligonucleotide hybridization. We examined mutational activation of exon and intron positions in preneoplastic foci, neoplastic nodules and carcinomas of the rat liver induced by diethylnitrosamine and G-A transitions in codon 12 of the H-*ras* gene in hepatocellular carcinomas induced by N-methyl-N-nitrosourea. No mutations were detected in the investigated hepatic lesions suggesting that neither mutations at codon 12, 13 and 61 of H-, K- and N-*ras* nor a mutation in the last intron of the H-*ras* gene are generally involved in initiation and progression of rat hepatocellular carcinomas. Besides the normal N-*ras* gene we amplified a variant gene which may be a pseudogene. In addition to these sequences (designated N-*ras*A and N-*ras*B) recently a third N-*ras* variant (N-*ras*C) with a mutation at codon 13 and putative oncogenic potential was found in normal liver of Fischer rats (McMahon et al. Proc Natl Acad Sci USA 87:1104-1108, 1990). N-*ras*C could not be detected in Wistar rats used in our experiments.

P 22

DISTRIBUTION AND PROGNOSTIC SIGNIFICANCE OF C-ERBB-2, C-KI-RAS, AND C-MYC PROTOONCOGENE (ONC) OVEREXPRESSION IN NON SMALL CELL LUNG CANCER (NSCLC)

J. Lorenz[1], T. Friedberg[2], W. Paulus[1], R. Perlinz[1], F. Oesch[2]
[1]III. Medizinische Klinik-Pneumologie, [2]Institut für Toxikologie, Universität Mainz

Activation of certain oncs by overexpression is associated with rapid proliferation and altered phenotype in vitro in some tumors. We therefore investigated the expression of c-erbB-2, c-Ki-ras, and c-myc in tumor and lung tissue biopsies of 45 patients (pts) with NSCLC. Lung tissue was available in 27 cases. We applied a RNA hybridisation technique with the use of S1-nuclease digestion of single stranded RNA. The clinical follow up of 36 pts undergoing surgical resection with curative intend was observed up to 21 months. The propability of disease free survival was estimated and groups without and with onc overexpression were compared using the logrank test. In only one lung sample a slight 2-fold overexpression of c-erbB-2 was seen. Tumor samples showed a slight (+,2 - 4-fold) overexpression of c-erbB-2 in 36% and a strong (++,>4-fold) overexpression in 29%. Overexpression of c-Ki-ras was observed in 32% (+) and 17% (++) and that of c-myc in 16% (+) and 27% (++). Some preference of c-erbB-2 and c-Ki-ras overexpression in adenocarcinoma (82% and 58% respectively) was observed, while c-myc was preferentially (72%) overexpressed in undifferentiated large cell carcinoma. Only c-erbB-2 overexpression had a significantly adverse impact on the disease free survival (p= o,o345).

T 1

HYDROGEN ION-MEDIATED ACTIVATION OF CYTOTOXIC DRUGS: A STRATEGY TO IMPROVE THE THERAPEUTIC INDEX OF ANTI-CANCER AGENTS

E. Jähde, K.-H. Glüsenkamp, L.F. Tietze*, and M.F. Rajewsky
Institute of Cell Biology (Cancer Research), University of Essen Medical School and *Institute of Organic Chemistry, University of Göttingen

Tumor-selective chemotherapy must be based on phenotypic differences that distinguish cancer cells from normals cells. It has been well established, both in animal and human tumors, that a metabolic hallmark of malignant cells, lactic acid production, can be exploited to increase the concentration of H^+ ions 5 - 10-fold selectively in tumor tissues. We are investigating whether such a transient modification of the internal milieu of malignant tumors can be used to improve the therapeutic index of anti-cancer agents by increasing their cytotoxicity selectively in malignant tissues IN VIVO. Three mechanisms of H^+ ion-mediated drug activation are being evaluated: (i) Increased reactivity (cytotoxicity) of alkylating drugs at reduced pH. For example, at pH 6.2 - the mean pH in tumors following glucose-mediated stimulation of lactic acid production - the cytotoxicity of "activated" cyclophosphamide on cultured malignant cells was potentiated by a factor of 200 as compared to control cells treated at pH 7.4. In addition, this increase in cytotoxicity was sufficient to fully compensate drug resistance in cyclophosphamide resistant cells. (ii) H^+ ion sensitive prodrugs of alkylating agents were designed which are non-toxic at pH 7.4. At low pH, however, these prodrugs decompose with liberation of the active alkylating species. For this approach ketalglycosides and carboxyamides were chosen as acid-labile (activating) moieties. At pH 6.2 the cytoxic effects of this class of agents, as measured by the survival rate of colony-forming cells, was increased by several orders of magnitude as compared to physiological pH. (iii) Our third approach aims at activation of non-toxic prodrugs by pH-dependent enzymatic reactions in conjunction with enzyme targeting by monoclonal antibodies. Prodrugs were synthesized which, in the presence of the corresponding enzymes, are not active at physiological pH. However, at "intratumoral" pH (6.2) the activity of the enzymes was increased resulting in dose-dependent cytotoxicity of the prodrugs.

T 2

Cisplatin-resistance associated karyotypic evolution from heteroploidy to near-diploidy in a rat ovarian tumor cell line assayed by flow cytometry and determination of chromosome number distribution

G. Chen[1], K.-J. Hutter[2], J. Bullerdiek[3] and W. J. Zeller[1]
[1]Institute of Toxicology and Chemotherapy, [2]Institute of Radiology and Pathophysiology, Applied Immunology, Flow Cytometry, German Cancer Research Center, 6900 Heidelberg, FRG; [3]Center of Human Genetics and Genetic Counselling, University of Bremen, 2800 Bremen 33, FRG.

In a rat ovarian tumor cell line a 33-fold resistance to DDP (O-342/DDP) was developed in vitro by continuous exposure of the parental cell line (O-342) to a stepwise increase of DDP concentration in the culture medium. Induction of resistance lasted about 6 months before sensitivity of both lines against cisplatin was determined by colony forming efficiency. Both cell lines had a similar growth rate with a doubling time of about 24 h in log phase growth in vitro. Development of resistance was accompanied by evolution of the karyotype from heteroploidy in chemosensitive O-342 cells to near-diploidy in resistant O-342/DDP cells as shown by chromosome number distribution. This finding was confirmed by measuring cellular DNA content using flow-cytometry analysis. In addition, flow karyotyping showed significant differences in chromosomal DNA contents between both cell lines. Our results suggest that the parental line O-342 consists of at least two subpopulations, a DDP-sensitive and a DDP-resistant one, corresponding to heteroploidy and near-diploidy, respectively. Continuous DDP-exposure of O-342 selectively killed the sensitive fraction, resulting in the karyotypic evolution observed.

T 3

THE STABLE PROSTACYCLIN ANALOGUE CICAPROST INHIBITS TUMOR METASTASES IN DIFFERENT TUMOR MODELS IN RODENTS

M. Schirner and M.R. Schneider
Research Laboratories of Schering AG Berlin

The concept of the metastatic tumor spread implies a potential therapeutic role for compounds which inhibit tumor cell-platelet aggregation. Compounds with antiaggregatory properties were shown to reduce tumor cell platelet aggregation - a crucial step in tumor dissemination. Therefore, we studied the effect of Cicaprost, a stable, orally active prostacyclin-analogue, on the number of tumor metastases in the M5076 reticulum sarcoma of the mouse and the R3327 MAT Lu prostate carcinoma of the rat. In the s.c.-implanted, spontaneously metastasizing M5076 reticulum sarcoma, the number of visible liver metastases was significantly inhibited by all three doses tested (0.1, 0.3 and 1.0 mg/kg). In the 1.0 mg/kg dose, Cicaprost reduced the number of metastases by 90% compared to the control. In the second test, Cicaprost decreased the number of liver metastases of i.v.-injected M5076 reticulum sarcoma cells when administered 3 or 6 hours before tumor implantation. No extrahepatic metastases were found in either treatment group. In the s.c.-implanted spontaneously metastasizing R3327 MAT Lu prostate carcinoma of the Cop rat, Cicaprost was given in a dose of 1.0 mg/kg p.o. daily throughout the experiment without removing the primary tumor. At the end of the experiment the number of lung metastases was reduced by more than 80% compared to the control. These data indicate that the stable prostacyclin analogue Cicaprost has pronounced antimetastatic activity in spontaneously as well as i.v.-injected metastasizing tumor models and therefore is a promising candidate for specific antimetastatic therapy.

T 4

Evidence for Hormone Receptor Mediated Action of an Androgen-linked Nitrosourea Derivative in MCF7 Cells

W. Hefter, P. Zhu, M. Gebelein, B. Naser-Hijazi, G. Eisenbrand, M.R. Berger
Institute of Toxicology and Chemotherapy
German Cancer Research Center, 6900 Heidelberg, F.R.G.

N-(2-hydroxyethyl)-N'-nitrosourea-succinato-dihydrotestosterone-17-ester (HECNU-suc-DHT) is a very effective cytostatic agent in the methylnitrosourea-induced rat mammary carcinoma. In vitro investigations were made to elucidate a possibly hormone receptor mediated mode of action of this compound. After 6 hours of incubation with 100 μM of the mixture of HECNU-suc + DHT the growth of MCF7 cells -determined at 4 days after treatment- was inhibited by only 27%, 50 μM had no inhibitory effect at all. HECNU-suc-DHT strongly inhibited cell growth by 90% and 79%, respectively, at the indicated concentrations.
Coincubation with estradiol (E_2) and dihydrotestosterone (DHT) for 4 hours antagonized the cell growth inhibition in a dose-dependend manner. 50 μM E_2 antagonized the cell growth inhibition of 20 μM HECNU-suc-DHT distincly from 39% to 9% and that of 50 μM from 79% to 61%. DHT was less effective than E_2. 50 μM DHT reduced the growth inhibition of 20 μM HECNU-suc-DHT from 37% to 19% and that of 50 μM from 66% to 53%.
The KB epidermoid carcinoma cell line showed a similarly strong inhibition of cell growth by HECNU-suc-DHT but the effect could not be competitively antagonized by either hormone.
Therefore we conclude that part of the growth inhibitory effect of HECNU-suc-DHT in the receptor positive human breast cancer cell line MCF7 is mediated by steroid hormone receptors, mainly the estrogen receptor.

T 5

MURINE MONOCLONAL ANTIBODIES AND A RECOMBINANT SINGLE CHAIN Fv FRAGMENT SPECIFIC FOR THE EXTRACELLULAR DOMAIN OF THE HUMAN c-erbB-2 PROTEIN: NOVEL APPROACHES TO TARGETED DRUG THERAPY.

Winfried Wels, Ina-Marie Harwerth, Bernd Groner and Nancy E. Hynes
Friedrich Miescher Institute, P.O. Box 2543
4002 Basel, Switzerland

The c-erbB-2 proto-oncogene encodes a transmembrane glycoprotein of 185 kd. This protein has structural homology with the growth factor receptor-tyrosine kinases and is most likely important for the growth or differentiation of specific epithelial cells. Enhanced expression of the gp185 c-erbB-2 protein is found in about 30% of human primary breast and ovarian cancers, making this protein an interesting target for directing cytotoxic reagents to these tumors. We have prepared a panel of monoclonal antibodies (MAbs) which specifically react with the extracellular domain of the c-erbB-2 protein. The MAbs were tested for their effects upon c-erbB-2 receptor internalization and upon anchorage dependent and independent growth of cells expressing elevated c-erbB-2 levels. The variable domains of one of the MAbs, FRP5, were cloned by reverse transcription of poly-A RNA from hybridoma cells and amplification of cDNA by the polymerase chain reaction (PCR). A fusion gene coding for a single chain Fv fragment was constructed and used for the expression of a 26 kd Fv in E.coli. The recombinant single chain Fv, like the original MAb, shows specific binding to the c-erbB-2 protein and to cells expressing high c-erbB-2 levels. Strategies for the construction of chimeric Fv constructs, encoding linked enzymes, have been evaluated.

T 6

Increased ADP-ribose transferase (ADPRT) activity in a DDP-resistant ovarian tumor cell line and its relation to DNA repair.

G. Chen and W.J. Zeller
Institute of Toxicology and Chemotherapy, German Cancer Research Center, 6900 Heidelberg, FRG

Serial determination of ADPRT activity following cisplatin (DDP) exposure in a DDP-resistant rat ovarian tumor cell line (O-342/DDP) and its parental counterpart (O-342) was carried out by measuring [^{3}H]-NAD (a substrate for ADPRT) incorporation. ADPRT activity in O-342/DDP cells was found to be 2.85 times as high as that in O-342 cells (839 vs. 294 dpm/10^6 cells) in 4 separate experiments. Following DDP exposure (20 μM, 2 h), ADPRT activity in O-342 cells was at first slightly inhibited, then stimulated with a maximum at 24 h (50 % increase vs. control), while in O-342/DDP cells the activity of this enzyme was continuously decreased at all time points following DDP exposure, although at 12 h it almost recovered to the control level (799 vs. 839 dpm/10^6 cells). Correspondingly, DNA single strand breaks (DNA-SSB), which are known to stimulate ADPRT activity, mostly occurred at 24 h in O-342 cells and at 12 h in O-342/DDP cells after DDP treatment as assayed by alkaline elution. Accordingly, 3-aminobenzamide (3AB), a well-known inhibitor of ADPRT, maximally inhibited ADPRT activity at 24 h and 12 h post DDP treatment in O-342 and O-342/DDP cells, respectively. Furthermore, 3AB was shown to significantly decrease DNA interstrand crosslinks (ISCL) at 24 h in O-342 cells and at 12 h in O-342/DDP cells following DDP treatment. Altogether, our results suggest that increased ADPRT activity and a change of DNA repair as shown by alkaline elution may be involved in development of DDP resistance.

T 7

In vivo Effect of Hexadecylphosphocholine on Methylnitrosourea (MNU)-induced transplanted mammary carcinoma in Rats

M. Reinhardt, P. Heyl, M.R. Berger and D. Schmähl†
German Cancer Research Center, Institute for Toxicology and Chemotherapy, Im Neuenheimer Feld 280, 6900 Heidelberg (FRG)

The new antineoplastic agent hexadecylphosphocholine (HPC) shows high antineoplastic activity in MNU-induced mammary carcinoma. In other tumor models, however, HPC has only a small effect or is inactive. The mechanism of action of HPC is probably membrane directed .

To elucidate this mechanism we transplanted MNU-induced mammary carcinomas serially to obtain sensitive and resistant tumors and characterized a possible change in sensitivity by cytoskeleton examination in all passages. Vimentin could clearly be detected in all passages whereas cytokeratin was marginally expressed at early passages, and appeared clearly only after repeated transplantations. In passages 5 and 10 the animals were treated with pulse doses of HPC once weekly for five weeks to determine the sensitivity to this agent. During therapy the body weight loss was used as indicator of toxicity and tumor volume served as parameter of therapy success. In the 5th passage HPC (112,5mg/kg body weight) effected a significant reduction of the tumor volume by about 50%, whereas in the 10th passage treatment with the same dose had no significant effect on tumor volume. This change in chemosensitivity was paralleled by a continuous acceleration of tumor growth, as characterized by the time of tumor manifestation following implantation of the tumor cell suspension: This time decreased from 15 weeks (passage 1) to 1 week (passage 10).

The selection pressure of passaging probably was responsible for the change in chemosensitivity of the transplanted tumor.

T 8

THROMBIN INDUCES ACTIVATION OF pp60^{c-src} PROTEIN TYROSINE KINASE IN HUMAN PLATELETS

P. Presek, U. Liebenhoff and D. Findik

Stimulation of human platelets by thrombin, the phorbolester TPA, collagen and other agonists causes a transcient increase in tyrosine phosphorylation of a variety of proteins. Changes in tyrosine phosphorylation occurred in three temporal waves. Proteins of 48-50, 55, 64-70, 95 and 115-130 kDa were affected. Looking for the kinase responsible for the enhanced tyrosine phosphorylation we studied pp60^{c-src}, the most abundant protein tyrosine kinase in platelets. pp60^{c-src} represents the cellular homologue of the transforming protein of Rous sarcoma virus. pp60^{c-src} is maximally stimulated 2-3 fold by thrombin in a time- and concentration-dependent manner. TPA and collagen were also effective. Analysis of immunoprecipitated pp60^{c-src} revealed an enhanced phosphory-lation of the protein due to stimulation. Distinct changes in pp60^{c-src} phosphorylation after thrombin treatment are discussed in relationship to already studied pathways of pp60^{c-src} stimulation. The present study focuses on a mechanism involved in platelet activation and demonstrates pp60^{c-src} function in mature cells additionally to its apparent role in differentiation processes.

Rudolf-Buchheim-Institut für Pharmakologie, Frankfurter Str. 107, 6300 Gießen, FRG

T 9

STUDY OF AN IN VITRO ASSAY TO PREDICT HUMAN BRAIN TUMOUR RADIOSENSITIVITY: CORRELATION BETWEEN AN IN VITRO RADIOSENSITIVITY ASSAY AND TREATMENT FOLLOW-UP

G. H. Hartmann[1], H. Fischer[1], C. Kliesch[1], G. Schackert[2], F. Albert[2], S.Kunze[2], M. Wannnenmacher[3]

[1] Deutsches Krebsforschungszentrum, Heidelberg, [2] Neurochirurgisches Zentrum und [3] Klinische Radiologie, Universitätsklinik Heidelberg

Recently, a great interest in laboratory assays which might predict tumor treatment response has grown. The question tackled in this work was: how important is intrinsic cellular radiosensitivity as an independent determinate of brain tumour response to radiation.

At 54 patients the radiosensitivity of brain tumours was determined by an in vitro assay using tissue specimens that are taken during surgery (1). This was done parallel to a postoperative radiotherapy. For each patient the results of both the in vitro assay and the clinical therapy are correlated by evaluation of his medical records.

Glial tumours, meningiomas and brain metastases of extracranial tumours were subject of our investigations. According to their in vitro reaction to x-ray doses from 30 to 90 Gy they were classified into a) sensitive, b) moderately sensitive and c) resistant tumours. The highest and mainly homogenous radioresistance was found in the group of the meningiomas. For comparison various specimens of the same meningioma were exposed to a 60 Gy single dose and to a fractionated scheme (5x12 Gy/day), respectively. A substantial difference could not be detected. However, a remarkable individual spread of radiosensitivity was found for glioblastomas. The same applies for grade II and III-gliomas.

Comparing experimental and clinical data, it appears that there is indeed a close correlation. For example, some patients showed recurrence of tumour only a few months after surgery and radiotherapy. All these tumours had already been conspicuous in the cell culture assay by an especially high radioresistance and rapid growth.

(1) Fischer et al., Acta Neurochir. (1987) 85:46-49

T 10

FIRST EXPERIENCES WITH INTRAOPERATIVE RADIATION THERAPY (IORT) FOR GASTRIC CANCER

J. Boese-Landgraf, E. Lorenz, D. Albrecht and M. Dulce; Klinikum Steglitz, Berlin.

In an effort to treat the advanced gastric cancer more successfully we combined standard surgical procedure with IORT (15Gy) of the compartment II plus post-operative external radiation therapy to a total dose of 60Gy. Nineteen of 21 patients had histologically an advanced cancer: 9 diffuse, 9 intestinal- and 1 intermediate type gastric carcinoma. In a follow-up period of 17 months 6 patients with a diffuse type received metastatic recurrence, none of the others. As intestinal-type gastric carcinoma tend to recur only locally in 30%, this type of carcinoma may profit from adjuvant radiation regimen.

T 11

Intraarterial Infusion of MMC, 5-FU for Recurrent Breast Cancer - A Phase II Study in 94 Patients

K.R. Aigner, N. Thiem, H. Müller, M. Leonardi
Dept. of Surgery, K.K.H Trostberg, FRG
(Head: K.R. Aigner)

In 94 patients with recurrent breast cancer subclavian artery infusion was performed via an implantable Jet Port catheter (PfM, Cologne, FRG). Chemotherapy consisting of MMC (14 mg) on day 1 and 5-Fluorouracil, (1000 mg) on day 2 - 5, infused over 60 min. each is given in four cycles in four weeks intervals.

Results: The overall response rate was 88 % (CR 30%, PR 45%, MR 17%). In the non pretreated group (n=21) there were 47.6 % CR and 33.3 % PR, in the gorup with prior chemotherapy and radiation the CR rate was only 13.9 % and 52.8 % respectively.

Pretreatment	CR	PR	MR	NR
None (n=21)	47.6 %	33.3 %	9.5 %	9.5 %
Chemotherapy (n=17)	41.2 %	29.4 %	17.6 %	11.8 %
Radiotherapy (n=20)	30.0 %	55.0 %	5.0 %	10.0 %
Radio- and Chemotherapy (n=36)	13.9 %	52.8 %	27.8 %	5.6 %

Subclavian artery infusion for locally recurrent breast cancer - response
related to pretreatment (n = 94)

Side effects and complications: Quality of life is not affected and pain relief is usually achieved after the first cycle. Three patients had spot like skin and soft tissue burn from drug streaming.

T 12

Isolated Pelvic Perfusion (IPP) For Nonresectable Pelvic Tumors

H. Müller, K.R. Aigner
Dept. of Surgery, K.K.H Trostberg, FRG
(Head: K.R. Aigner)

In an attempt to incrase locoregional drug exposure in pelvic tumors the aorta and vena cava were cannulated just above the bifurkation and perfused at a low flow rate (100ml/min.) for 60 min. using a heart lung machine (HLM). In order to keep the perfused volume minimal Esmarch bandages were placed around both roots of the limbs.

Material: 36 isolated pelvic perfusions were carried out in 33 patients (3 second perfusions) with nonresectable pelvic tumors. (19 rectum cancers, 4 melanomas, 6 gynecologic tumors, 4 sarcomas). A three drug combination was used in rectum cancer patients (MMC, 5-FU, ACNU), in melanomas (MMC, CDDP, ACNU) and in sarcomas (MMC, CDDP, L-Pam), whereas gynecologic tumors were treated with a two drug combination (MMC, CDDP). In 6 cases with recurrent rectum cancer we used Mitoxantron as a single agent.

Results: Mitoxantron and ACNU pharmacokinetics showed a steep increase of regional drug exposure compared to systemic plasmalevels (20:1). Medium plasma half life was 11.8 min. and 11.4 min. respectively.

According to CT-scan and tumormarker levels there were 2/33 CR (1 rectal-, 1 anal carcinoma) 24/33 PR (19 rectal carcinomas, 4 melanomas, 3 gynecologic tumors, 2 sarcomas), 1/33 MR (1 rectal carcinoma, 1/33 SD (1 sarcoma) and 5/33 NR. Resectability could be achieved in 12 cases out of 29 patients.

Complications: After the operation three patients had bleeding complications and one had ileofemoral venous thrombosis. Transient myelosuppression was observed in 6 cases.

T 13

A 3D-Model to Study Interactions (Drug-Drug, Drug-Radiation) for Sequential Modality Combination - Therapy in vitro

U. Bogdahn, A. Justh, D. Drenkard, M. Lutz , R. Apfel, C. Behl - Dptms of Neurology, Univ. of Würzburg, F.R.G. and Internal Medicine, Univ. Tübingen, F.R.G.

Combination chemotherapy is widely employed in clinical oncology, however, there is no generally accepted model to evaluate individual tumour susceptibility to a given drug combination regime resp. to a combination of radio- and chemotherapy. We therefore investigated drug interaction of *IFOSFAMID (4-Hydroxy-Peroxy-Ifosfamid)* and *ACNU* and *IFOSFAMID* and radiotherapy in a recently developed in vitro model of paired sequential combination therapy. A longterm standard (6,3-^{3}H)-Thymidine-incorporation assay employing a liquid scintillation counting protocol was selected to assess human tumour sensitivity. In vitro drug exposures were derived from correlating in vivo-(systemic and CNS-) and in vitro-pharmacokinetic drug parameters. In combination experiments tumour cells were treated sequentially by the 2 drugs in both sequences: drug exposures were calculated for 2 hrs, in between treatments a 1 hour interval without drug present was installed. "Cut-off" concentrations (maximum in vitro exposure doses) were 1.74 µM (primary CNS-tumours 0.58 µM) for *IFOSFAMID* and 5.4 µM (CNS: 1.33 µM) for *ACNU*. Dose response relations were derived from isotope incorporation rates after cells had grown for approx. 5 population doubling times. Sequential radio-/chemotherapy was performed accordingly: radiation doses included exponential dose-intervals from 0.5 to 3.25 Gy. Combination therapy response surfaces were derived from these in vitro data , allowing isobole analysis of putative sub-, supra- and additive combination therapy effects with a 3D model. Isoboles may be constructed at any effect level and in individual combination directions. Analysing the results of *3 glioblastomas*, 3 *bronchial carcinomas (brain metastases)*, 3 *malignant melanomas* , 2 *hypernephroid carcinomas* for drug combinations we conclude, that drug combination chemotherapy effects at the cellular level may be extremely heterogeneous: It is hypothesized that clinical effects of drug combinations may be optimized by drug modelling depending on tumour histology, specific individually active substances and individually selected drug sequences. Finally, it is feasible to analyse for drug-radiotherapy interaction with the proposed model.

T 14

COMPUTER AIDED DRUG DESIGN (CADD): AIDS TO GET A BETTER UNDERSTANDING OF A NEW PORPHYRINE DERIVATIVE P_4P-mD FOR TUMOR DIAGNOSIS AND PHOTODYNAMIC THERAPY

E. Lang, C.W. v.d. Lieth, G. Graschew[1], H. Sinn[2]

Deutsches Krebsforschungszentrum Heidelberg, Zentrale Spektroskopie
[1] Chirurgische Universitätsklinik Heidelberg
[2] Deutsches Krebsforschungszentrum Heidelberg

Porphyrins can be used as photosensitizers cumulating in tumors and making them accessible to laser light-induced fluorescence diagnosis and photodynamic therapy. The aim of recent investigations was to find substances which possess high local specifity, a low side effect rate and a good photosensitizing effect. The Cambridge Crystallographic Data Files (CCDF) are worldwide the largest collection of 3-dimensional coordinates and certain properties of substances which have been investigated by X-ray diffraction. Over thirty tetraphenyl porphyrin derivatives have been extracted from the CCDF by means of a powerful substructure search routine developed at the department of spectroscopy of the DKFZ which allows to retrieve substances with similar structures. The applicability of the tetraphenyl porphyrins bound to the carrier system PEOM could be estimated according to their structural properties. Geometric analyses and molecular dynamics have been performed with the set of retrieved structures. It could be shown that the porphyrin skeleton is not conjugated with the phenyl substituents and that it is very stable. The phenyl rings show steric hindrance and are not rotatable. CADD methods could give aids to design a porphyrin derivative which is stable (low bleaching), can be covalently bound to the carrier system PEOM and accumulates at a higher level in the malignant tissue than most photosensitizers do.

T 15

Technique of sample extraction and High-Pressure-Liquid-Chromatography (HPLC) for comparative pharmacokinetic studies with estradiol linked nitrosoureas.

B.R. Betsch, B. Spiegelhalder, M.R. Berger, D. Schmähl

Institute of Toxicology and Chemotherapy, German Cancer Research Center, 6900 Heidelberg

Estradiol-linked nitrosoureas are offering new perspectives in the treatment of estradiol receptor positive tumor tissues. For pharmacokinetic studies a new method was developed for the simultaneous analysis of the estradiol-linked nitrosoureas N'-(2-chloroethyl)-N'-nitroso-carbamoyl-L-alanine-estradiol-17-amide (CNC-ala-E_2-A) and N'-(2-chloroethyl)-N-nitroso-carbamoyl-L-alanine-estradiol-17-ester (CNC-ala-E_2-E) with their respective metabolite N'-(2-chloroethyl)-N-nitroso-carbamoyl-L-alanine (CNC-ala).

All three compounds were extracted from plasma with C_{18} silicagel reversed phase cartriges (Baker, Groß-Gerau, F.R.G). The clean up technique delivered clear samples only slightly contaminated with the biological matrix. The recovery from plasma was 70 ±5% for CNC-ala and 75 ±5% for the estradiol-linked compounds.

The analysis was carried out by means of HPLC (HP 1090 Hewlett Packard, Waldbronn, F.R.G.). To allow a simultaneous detection of CNC-ala and CNC-ala-E_2-A or CNC-ala-E_2-E, respectively, the unlinked single agent CNC-ala was derivated with diazomethane prior to analysis. The compounds were detected by an UV detector at 230 nm after separation over a spherisorb-C_{18} column, 25 x 0.4 cm/5 µ (Latek, Heidelberg, F.R.G.) with a solvent gradient (1 ml/min) of methanol=m, acetonitril=a, and water=w (start: m/a/w/=10/20/70; end: m/a/w/=80/0/20). The limit of detection was 1 nmol/ml. The described technique provides a selective, highly sensitive and well reproducible procedure for the analysis of estradiol-linked nitrosoureas.

T 16

DNA STRAND BREAKS CAUSED BY CHEMOTHERAPY WITH ALKYLATING AGENTS IN HUMANS

Jan Georg Hengstler, Jürgen Fuchs, Franz Oesch;
Institut für Toxikologie, Universität Mainz, Obere Zahlbacherstr. 67, D-6500 Mainz

The therapy with alkylating agents caused DNA strand breaks in mononuclear blood cells of cancer patients which was measured as an accelerated elution rate with the alkaline filter elution method.
An investigation of 18 patients suffering from different types of cancer showed an increased elution rate of 128% on the average in those patients receiving alkylating agents compared to the control group. The difference was highly significant with $p < 0.005$ (U-test). In the group of patients who received non alkylating antitumor agents no increase in DNA strand breaks could be detected.
In a successive study DNA strand breaks and DNA cross links were investigated prior to and after a therapy cycle in 15 ovarian carcinoma patients who received therapy with alkylating agents (CP-scheme: cyclophosphamide and carboplatin). The patients showed an increased elution rate of 37% compared to controls already before the current chemotherapy cycle. At the end of the actual cycle of therapy an acceleration of the elution rate of 157% on the average compared to controls was found. The amount of DNA cross links was also increased at the end of the therapy cycle.
The patients showed different responses after drug intake. While some patients showed nearly no acceleration of the elution rate, others showed an acceleration up to 400%. Monitoring the course of the disease in 6 of these patients indicated that a strong acceleration of the elution rate during the therapy is possibly linked to the success of this cancer treatment measured as a decrease in the tumor marker CA12-5 to the normal level.

T 17

INFLUENCE OF A DNA-CROSSLINKING AGENT WITH AFFINITY TO THE ANDROGEN RECEPTOR ON THE MOUSE MAMMARY TUMOR VIRUS HORMONE RESPONSE ELEMENT IN MAMMARY CARCINOMA CELLS

F.J. Busch[1], A.B.C. Cato[2], G. Eisenbrand[1]
[1] Univ. Kaiserslautern, FR Lebensmittelchem/Umw.tox. P.O.B. 3049, D-6750 Kaiserslautern, [2]Kernforsch., Inst. f. Gen. u. Tox. v. Spaltst., P.O.B. 3640, D-7500 Karlsr., FRG.

1-(2-chloroethyl)-1-nitrosocarbamoyl-L-alanine-dihydrotestosterone-17-ester (CNC-ala-DHT) is an antitumor drug with affinity to the androgen receptor highly effective in experimental mammary carcinomas. Interaction with the steroid hormone response element (HRE) was studied in T 47-D cells stably cotransfected with mouse mammary tumor virus long terminal repeat sequences (MMTV-LTR) linked to the chloramphenicol acetyltransferase (CAT) gene and a chimeric construct containing the SV 40 promotor linked to the neomycin resistance gene. Sequences within the LTR region of MMTV confer androgen inducibility to the MMTV-LTR promotor. CAT activity and transcription at the MMTV-Ltr region were measured. CNC-ala-DHT (10^{-4}M; 15 min) induced a strongly decreased expression at the MMTV-LTR promotor in the CAT-assay compared to DHT. Simultaneous treatment with an equimolar dose of DHT resulted in a slight increase in CAT-activity. Likewise, transcription at the MMTV-LTR promotor was inhibited by 10^{-4}M CNC-ala-DHT and there was no substantial increase after simultaneous incubation with 10^{-4}M DHT. In contrast, incubation with a mixture of CNC-ala and DHT (10^{-4}M; 15 min) led to marked overinduction of MMTV transcription in comparison to 10^{-4}M DHT alone. The results suggest a specific interaction between CNC-ala-DHT and the LTR region or with the hormone receptor.

T 18

ATTEMPTS TO EXPLOIT PLASMIN ACTIVATOR ACTIVITY OF TUMOUR CELLS FOR A BETTER TARGETTING OF DNA CROSSLINKING AGENTS

G. Schied, W. Tang, G. Eisenbrand
Dept. of Food Chemistry and Environm. Toxicology, University of Kaiserslautern, D-6750 Kaiserslautern, FRG

A series of malignant tumor cell lines as well as human and animal solid tumors secrete substantially increased amounts of plasminogen-activators compared to normal tissues. As a consequence, an enhanced plasmin activity is formed from plasminogen in the vicinity of tumour cells. We try to achieve a more selective antitumour activity of alkylating agents by linking them to plasmin substrates. New aminoalkyl-chloroethylnitrosoureas (aminoalkyl-CNUs) and their conjugates with appropriate plasmin substrates such as D-val-leu-lys, pyr-phe-lys, Z-ala-ala-lys, and D-val-phe-lys were synthesized. DNA crosslinking activity of 2-aminoethyl-CNU and its conjugate D-alanyl-phenylalanyl-lysyl-aminoethyl-CNU has been tested in Namalva cells, a human lymphoblastoid cell line, with or without added plasmin. The free aminoethyl-CNU (10^{-4}M) effected a crosslinking activity (rad equivalent) of 41 whereas the conjugate showed only an activity of 15 (10^{-4}M). Addition of plasmin (0.1 U/ml) elevated the crosslinking activity for both, the free aminoethyl-CNU and its conjugate to 95 and 91 (10^{-4}M), respectively. Since incubation with plasmin lead also to an increased uptake of trypan blue, we conclude that proteolytic enhancement of membrane permeability also contributes to the observed effects. Further investigations using cells with high plasminogen activator activity are under way.

T 19

GROWTH FACTOR EXPRESSION IN HUMAN MALIGNANT GLIOMAS AFTER IN VITRO BCNU CHEMOTHERAPY

BOGDAHN, U. Richter B., Behl C., Apfel R., Drenkard D.,
Dept. of Neurology, Würzburg Univ., D-8700 Würzburg, F.R.G.

Three human glioblastoma early passage cultures HTZ-17, HTZ-146, HTZ-194A, were treated for three treatment blocks with an average maximal clinical exposure dose of BCNU (8.1µM for 2hrs). The chemosensitivity of these cells for BCNU was determined for each tumor before, after therapy and for untreated controls. mRNA expression levels for PDGF-A-chain, -B-chain, TGF-α, bFGF, TGF-β_1, TGF-β_2, PDGF-receptor A -type and B-type , and EGF-receptor in respective cell populations were determined by northern hybridizations. Although chemosensitivity remained exactly unchanged in all 3 cultures, HTZ-17 displayed an increase in TGF-β_1 mRNA, HTZ-146 an increase in PDGF-A-chain mRNA, and HTZ-194A in bFG-mRNA-levels in treated versus untreated cells. This indicates a putative role of TGF-β_1, bFGF and PDGF in glioma cells surviving BCNU-chemotherapy. We conclude that individual malignant brain tumors may react to nitrosourea chemotherapy with an individual patern of adapted growth factor mRNA-expression.

T 20

EFFECT OF HUMAN TUMOR NECROSIS FACTOR-α AND A HIGH-MOLECULAR FRACTION OF PLANT-DERIVED EXTRACTS ON GROWTH AND CELLULAR COMPOSITION OF A RAT ASCITES TUMOR

C. Schaefer, D. K. Kelleher and P. Vaupel
Institute of Physiology and Pathophysiology, University of Mainz, D-6500 Mainz, Germany

The impact of recombinant human tumor necrosis factor-α (rhuTNF-α) and a high-molecular fraction of the combined plant-derived extracts (HMFPE) from Baptisia tinctoria, Echinacea purpurea, and Thuja occidentalis on growth of a rat ascites tumor model (DS-carcinosarcoma) was studied. Tumors were implanted by i.p. injection of 2 x 10^8 tumor cells and TNF-α(1 mg kg^{-1}) and HMFPE (10 mg kg^{-1}) were administered every 12 h i.p., starting 8 h after tumor implantation. Injections of the appropriate vehicle were given to control animals. After 7 days the animals were sacrificed and ascites volume, cytocrit, and cell count per unit ascites volume determined. Cell smears were prepared (Romanowsky-Giemsa stain) and differentiated into intact tumor cells, tumor cell ghosts, monocytes, lymphocytes and polymorphonuclear leukocytes.
Ascites weight was significantly reduced following TNF-α or HMFPE treatment. Total cell count was found to be significantly reduced in TNF-α-treated animals whereas upon HMFPE treatment no significant change was seen. Growth changes were accompanied by changes in the proportion of different cell types in the ascites. The proportion of viable tumor cells was significantly reduced by 30% upon TNF-α or by 57% with HMFPE. However, different effects on the leukocyte proportion were seen with these agents. TNF-α produced significant increases in the proportion of monocytes, lymphocytes and polymorphonuclear leukocytes. HMFPE produced a significant increase only in the proportion of polymorphonuclear leukocytes.

TNF-α was provided by Knoll AG (Ludwigshafen), HMFPE was donated by Schaper & Brümmer (Salzgitter).

T 21

EFFECT OF RECOMBINANT HUMAN ERYTHROPOIETIN (rhEpo) ON THE GROWTH OF HUMAN TUMOR CELL LINES IN VITRO

D. Mundt, M.R. Berger, Institut für Toxikologie und Chemotherapie, DKFZ, Heidelberg

Several growth factors are well-known for their stimulation of human tumor cell lines in vitro. We tested the effect of rhEpo on the proliferation of human skin, kidney, mamma, liver, colon, and lung tumors with the microculture tetrazolium assay (MTT-Assay). A range of 1, 10, 100, 1000, and 5000 units of rhEpo (Boehringer Mannheim GmbH) per ml containing 10 x 10^4 cells were incubated for three to six days including generally two cell doubling times. None of the rhEpo dosages had any effect on the growth of any of the tested human tumor cells, neither in a stimulatory nor an inhibitory way. Those of our cell lines which form colonies in the Hamburger-Salmon-Assay were also incubated with different dosages (1-5000 U/assay). Colonies were counted after one, two, and three weeks of incubation. There was no colony-stimulating effect on the cells by any dosage of rhEpo. We saw a slight colony reduction after three weeks of incubation, probably due to insufficient nutrition after that period of time. The results are of clinical relevance in that rhEpo may be an untoxic adjuvant therapy of tumor- and chemotherapy-associated anemia.

T 22

Tolerability and efficacy of sequential therapy with rec. human IL-3 and rec. GM-CSF after intensive chemotherapy in patients with ovarian and breast cancer

H. H. Bartsch*, H. Meden, M. Meyer*, K. Vehmeyer*, R. Mull***, and W. Kuhn****
*Abt. Hämatologie/Onkologie Univ. Göttingen, ** Univ. Frauenklinik Göttingen, *** Sandoz Pharma Ltd., Basel, Schweiz**

Hemopoietic growth factors such as IL-3 (multi-CSF), GM-CSF and G-CSF were investigated in clinical phase-I and phase-II trials for their efficacy to reduce chemotherapy induced hematologic toxicity in patients with malignant diseases. Up to now there is little information about the combined effect of CSF's which act on early (IL-3) and determinated (GM-CSF) progenitor cells. We started a clinical phase-I trial to investigate the tolerability and efficacy of rhIL-3 alone, given as 24 hours cont. infusion for 5 days or the sequential application of rhIL-3 (same dose + schedule) and rhGM-CSF as s.c. injection for 7 days. Pts. with advanced ovarian and breast cancer receive cyclophosphamid 500 mg/m^2, epirubicin 50 mg/m^2 and carboplatin 300 mg/m^2. During the second treatment cycle pts. receive additional rhIL-3 alone (group 1 + 3) or sequential rhIL-3 and rhGM-CSF (group 2 + 4). rhIL-3 doses are 0,5 mcg/kg and 2,0 mcg/kg while rhGM-CSF dose is 3,0 mcg/kg. Seven pts. are included into each treatment group from whom one pat. receives placebo infusion. Up to now 18 pts. are enrolled in this trial and 14 pts. have completed the study. There were no clinical side effects according to WHO-criteria observed during rhIL-3 infusion in the low dose group either alone or during sequential therapy with rhGM-CSF. We found a clear increases in the number of eosinophil granulocytes and an increased recovery of platelets in group 1. After sequential application of rhIL-3 and rhGM-CSF there was also a significant increase in reticulocytes documented. We also measured a remarkable increase of circulating peripheral stem cells with a max. no. of 600 colonies/ml on day 14. Immunphenotyping of peripheral lymphocytes revealed evidence of IL-3 induced expression of IL-2 receptors.

T 23

STEROID-LINKED ANTHRACYCLINES: RECEPTOR BINDING AFFINITY AND ANTITUMOR ACTIVITY

S.Hoffmann, W.Tang, D.Berger*, H.Fiebig*, G.Eisenbrand
Depart. of Food Chemistry and Environm. Toxicology, University of Kaiserslautern, D-6750 Kaiserslautern, FRG
* Depart. of Internal Medicine, University of Freiburg, D-7800 Freiburg, FRG

Steroid-linked adriamycin, daunomycin and epirubicin have been synthesized in an attempt to achieve a better targetted drug delivery to hormone-receptor containing tumor tissues. Estradiol (E_2) and dihydrotestosterone (DHT) have been selected as carrier molecules and N-hemisuccinato-oligopeptides with alternating leucine and alanine sequences as spacers. The C-terminal amino acid was connected to the 3'-NH_2 group of anthracyclines and the N-terminus to the 17-OH group of steroids by a succinato ester bond. Relative binding affinities (RBA) of the E_2-conjugates to estrogen receptor from calf uterus cytosol were very low (0.1 to 0.2). The DHT-conjugates, however had RBA-values to androgen receptor from 0.5 to 2.0, depending on number and nature of amino acids. In eight human xenograft cell lines and P 388 mouse leukemia cells adriamycin-leu-ala-succ-DHT was only active at concentration as high as 2µg/ml, whereas unlinked adriamycin was active already at 0.02µg/ml. Similarly poor activity was found in human xenograft cell lines and P 388 mouse leukemia cells with 3 further adriamycin conjugates having various spacers. The poor antineoplastic effectiveness of the conjugates appears to result from their high stability.

T 24

PHARMACOKINETICS OF ANTICANCEROUS OXYSTEROLS

Ch. Moog, B. Bertram, N. Frank, B. Luu*, D. Schmähl
Institut für Toxikologie und Chemotherapie, Deutsches Krebsforschungszentrum, Heidelberg, * CNRS, URA 31, Strasbourg

Oxysterols, a family of naturally occurring substances have been shown to possess several biological activities. In particular some of them are toxic towards tumor cells in vitro. In vivo studies were difficult to carry out because of solubility problems. Therefore new hydrosoluble derivatives of oxysterols (phosphodiesters of oxysterols linked to nucleosides) have been synthesized. These prodrugs were active in vitro and in vivo.

The pharmacokinetics of one of these compounds, i. e. the sodium salt of 3-(7ß-hydroxy-cholesteryl)-5'-(2'-deoxyuridylyl)-monophosphate (JB 69) has been studied. Male Sprague-Dawley rats weighing 100 g were used. JB 69 was diluted in phosphate buffer pH 7.2 and applicated i.p. (50 mg/kg). After different time points (5', 1h, 4h, 24h) blood was taken. After killing the animals, liver and kidneys were removed, prepared and extracted for hplc examination.

JB 69 is stable in the organism for at least 1 h as it could be isolated from the liver in 10 % yield. JB 69 is supposed to be hydrolysed in vivo to release 7ß-hydroxycholesterol. Preliminary results showed that this product of hydrolysis is indeed formed in vitro, but until now we did not succeed to prove such a metabolic pathway in vivo. Further experiments are underway.

T 25

Experimental antitumor activity of new metal complex compounds.

S. Frühauf, B. K. Keppler and W. J. Zeller

Institute of Toxicology and Chemotherapy, German Cancer Research Center, 6900 Heidelberg, Federal Republic of Germany.

Eight new metal complex compounds (3 platinum (Pt)-, 1 titanium (Ti)-, 4 ruthenium (Ru)-derivatives) were tested in a cisplatin (DDP)-sensitive (O-342) and a DDP-resistant (O-342/DDP) ovarian tumor line using the clonogenic assay. In O-342, CTDP was the most active new Pt-compound resulting in a % control plating efficiency (CPE) (± SE) of 1±1, 12±8 and 40±21 following continuous exposure (CE) to 10, 1 and 0,1 µM, respectively; it was comparable to the activity of DDP at equimolar concentration. In O-342/DDP, CTDP (10 µM) effected a % CPE of 18±8 (equimolar concentration of DDP: 26±9). The Ti-compound budotitane was promising at 100 µM (short-term exposure) in both lines; at this concentration, activity in the resistant line was higher than in sensitive line (7±7 vs. 34±17 % CPE at 100 µM). All Ru-complexes were higher active in O-342/DDP than in O-342; ICR was the most active candidate. Following CE of O-342/DDP cells to 10 µM ICR, % CPE was reduced to 18±4. Altogether, the results obtained with CTDP, budotitane and ICR warrant further investigation of these compounds.

T 26

Efficacy of two new platinum-linked phosphonates in the treatment of colorectal cancer of the rat and in vitro in two human colon carcinoma cell lines.

A. Galeano, M.R. Berger, B.K. Keppler
Institute of Toxikology and Chemotherapy, German Cancer Research Center, 6900 Heidelberg, FRG

Two new platinum-containing phosphonate compounds, cis-diammine[nitrilotris(methylphosphonato)(2-)-O^1,N^1]platinum II, (AMDP) and cis-cyclohexane-1,2-diamine[nitrilotris(methylphosphonato)(2-)-O^1,N^1] (DADP) were investigated in acetoxymethylmethylnitrosamine induced colorectal rat adenocarcinomas as well as in two human colon cancer cell lines (SW707 and SW948) in vitro. In the in vivo model the two compounds, AMDP and DADP, were administered at doses of 8 and 13 mg/kg i.v. as well as at 16 and 26 mg/kg p.o., twice/weekly, for 10 weeks, respectively. AMDP caused with both dosages a higher antitumor effect but also showed a more intensive toxicity; DADP on the other hand showed also significant tumor volume inhibition with both modes of application and low toxicity. The in vitro assays were performed with two cell lines derived from human colorectal adenocarcinomas. Utilizing both microculture tetrazolium test (MTT) and the Coulter Counter method, AMDP (IC_{50}=15 and 30 µg/ml in SW707 and SW948) was more effective than DADP (IC_{50}=250 and 400 µg/ml in SW707 and SW948) in inhibiting the cell growth at doses which might be realistic in vivo. These results correspond to those obtained in vivo and warrant further investigations with compounds of this class to elucidate their role in the treatment of colorectal cancer.

T 27

SENSITIVITY PATTERN OF DIFFERENT OSTEOSARCOMA LUNG METASTASIS CELL LINES EXPOSED TO NEW PLATINUM CONTAINING PHOSPHONIC COMPLEXES IN-VITRO.

P. Valenzuela-Paz [1], T. Klenner [1], B. K. Keppler [2]
[1] Institute of Toxicology and Chemotherapy, German Cancer Research Center; [2] Institute of Inorganic Chemistry, University of Heidelberg, 6900 Heidelberg, FRG

Two tumorigenic cell lines (C 42, C 39) and a non-tumorigenic one (C 10) of a lung metastasis from an osteosarcoma bearing rat were isolated. Their doubling times in vitro during log-phase were 18 h (C 42), 37h (C 39) and 22h (C 10). The exposure to different platinum-containing phosphonic complexes (AMDP: cis-Diammine[nitrilotris(methylphosphonato)(2-)-O^1,N^1]platinum (II), IMD: cis-Diammine-[iminobis(methylphosphonato)(2-)-O^1,N^1]platinum (II)) and their diaminocyclohexane analogs (DADP: cis-Cyclohexane-1,2-diamine-[nitrilotris(methylphosphonato)(2-)-O^1,N^1]platinum (II), DIMD: cis-Cyclohexane-1,2-diamine-[iminobis(methylphosphonato)(2-)-O^1,N^1]-platinum (II)) lasted either 4h or 24h using concentrations of 2.5 to 10 x 10^{-5} M. The compounds AMDP and IMD were effective in all three cell lines; in a time- and concentration dependent manner in C 39 and C 10 with a maximum inhibition of 80% (C39, AMDP) and 89% (C10, AMDP) at day 5. The inhibitory effect of AMDP and IMD on cell proliferation of C 42 was to a large extent independent from time and concentration of exposure with a maximum of 98% (AMDP) In contrast, the diaminocyclohexane analogs were effective only in C 42. Viability was significantly reduced in C 42 by AMDP and IMD and in C 10 only by AMDP. In conclusion: These results elucidate the high potency of the new platinum containing phosphonic complexes in inhibiting proliferation of osteosarcoma lung metastases cell lines and the structure-activity relationship in respect to different growth pattern.

T 28

α,ω-Bisacylphosphonates: New Drugs interacting with Hydroxyapatite and inhibiting tumor-induced Osteolysis.

H. Stadler [1], T. Klenner [1], G. Golomb [2], E. Breuer [3]

[1] Institute of Toxicology and Chemotherapy, German Cancer Research Center, 6900 Heidelberg, FRG; [2] Department of Pharmacy, [3] Department of Pharmaceutical Chemistry, School of Pharmacy, Hebrew University, Jerusalem 91120, Israel

Bisphosphonates are used clinically for a number of important bone/calcium metabolism related diseases. Although the geminal bisphosphonates are potent drugs for these indications their use is hindered by lack of selectivity and side-effects, e.g. when treating patients orally. Bisacylphosphonates, such as AdBP (disodium dihydrogen adipoylphosphonate) and SuBP (disodium dihydrogen suberoylphosphonate), have been synthesized and found to affect hydroxyapatite formation and dissolution in-vitro and to inhibit tumor-induced osteolysis in the Walkercarcinosarcoma rat model similar to the geminal bisphosphonates, e.g. ABP (disodium hydroxypropyl-bisphosphonate). In contrast, the dideoxo bisphosphonate HBP (lacking the functional oxo-groups), as negative control, showed no activity. In detail, the efficacy was assessed by x-ray examination at day 10 after the transplantation (p. tr.) using an arbitrary scale. Compounds were administrated on day 3 till day 5 p.tr. in single doses: 0,27 mmol/kg (A), 0,41 mmol/kg (B), 0,61 mmol/kg (C). When taking the mean osteolysis of controls as 100%, the treatment effect can be calculated as follows:

AdBP: A - 108%,	B - 84%,	C - 92%;
SuBP: A - 86%,	B - 36%,	C - 59%;
HBP: C - 140%;	ABP (0,05 mmol/kg) - 45%	

The bisacylphosphonates possess lower toxicity and their calcium complexes/salts have improved solubility properties. In conclusion: These are the first instances of non-geminal [P-$(C)_n$-P, n≥2] bisphosphonates that have been reported to be highly active in inhibiting tumor-induced osteolysis.

T 29

COMPARISON BETWEEN INTRA ARTERIAL (i.a.) AND INTRAVENOUSLY (i.v.) 5'DEOXY-5-FLUOROURIDINE (5'dFUR) ADMINISTRATION IN THE TREATMENT OF DS-SARCOMA AFTER I.M. TRANSPLANTATION IN RATS.

B. Naser-Hijazi, M. Gebelein, W. Hefter and M.R. Berger
Institut für Toxikologie und Chemotherapie
Deutsches Krebsforschungszentrum, INF 280, 6900 Heidelberg

5'dFUR is one of the 5-fluorouracil metabolites and has been used in the therapy of liver metastases from colorectal carcinoma. The influence of infusion time and dose on the anticancer efficacy of 5'dFUR was investigated after variation of the administration mode (i.a. and i.v. therapy).

99 SD-rats bearing the transplanted DS-Sarcoma i.m. in the right thigh were randomly distributed in 25 groups. 5'dFUR was administered via catheter into the iliac artery (loco-regional; 54 rats) or the iliolumbal vein (systemic; 45 rats). Treatment began 6 days after tumor implantation and lasted five days. The duration of administration (bolus, 1-h, 5-h and 24-h) and the dose (47.3, 94.6 and 189.2 mg/kg) were varied. The change in median tumor volume following 5'dFUR treatment served as indicator of therapy response.

After end of therapy we observed either tumor remission or progression. Following i.a. therapy there were more cases of complete remission (40%) than following i.v. therapy (27%). The long administration times (5-h and 24-h infusions) were more effective than bolus injection and 1-h infusion time. The data concerning median tumor volume and survival time suggest that a therapeutic improvement can be obtained after loco-regional chemotherapy.

T 30

TUMOR INHIBITING EFFECT OF A NEW CYCLOBUTANE-PLATINUM COMPLEX (D-19466) IN THE TRANSPLANTABLE OSTEOSARCOMA OF THE RAT.

T. Klenner [1], R. Voegeli [2], P. Hilgard [2]

[1] Institute of Toxicology and Chemotherapy, German Cancer Research Center, 6900 Heidelberg, FRG; [2] ASTA Pharma AG, POB 100105, 6000 Frankfurt, FRG

D-19466, a new cyclobutane-platinum complex has shown high antineoplastic activity in different in-vitro cell lines and a number of rodent tumors and human xenografts in-vivo. These results and the lack of nephrotoxicity, which is dose-limiting in clinical use of cisplatin (CDDP), has prompted further investigation in solid tumors, as the transplantable osteosarcoma of the rat. This tumor is characterized by bone-matrix formation, regular dissemination to the lungs and slow growth pattern. D-19466 was given i.v. twice a week for three weeks with a maximum total dose of 23.84 mg/kg (A), 11.94 mg/kg (B) and 5.94 mg/kg (C) versus CDDP 9 mg/kg as positive control. The tumor inhibiting effect was assessed by T/C% values at the end of the treatment period: D-19466 A: 33%, B: 42%, C: 50%, CDDP: 33%. Mortality after the treatment was found only in the CDDP treated group (25%) and the controls (12.5%). The body weight of the treated animals developed similar to that of the controls.
In conclusion: D-19466 is as potent as CDDP in inhibiting the growth of the solid osteosarcoma without being nephrotoxic. Even better results should be obtainable with D-19466 as the maximally tolerable dose was not attained in this study.

T 31

FURTHER CHARACTERIZATION OF D-19466, A PLATINUM COMPLEX WITH ANTICANCER EFFICACY

R. Voegeli[1], H.R. Nowrousian[2], P. Hilgard[1], P. Aulenbacher[1], H. Fiebig[3], B. Winterhalter[3]

Lactato-O,O'-1,2-bis(aminomethyl)cyclobutane-platinum(II) (D-19466) has been identified as a platinum complex with high anticancer efficacy. It is lacking the nephrotoxic potential of cisplatin, its main adverse side effects being its hematological toxicity comparable to that of carboplatin.
D-19466 showed excellent activity superior to that of cisplatin and carboplatin in the murine P388 and L1210-leukemia models after single or multiple intraperitoneal treatment. Similarly positive results could be obtained in the treatment of the Ehrlich Ascites carcinoma model.
D-19466 was also evaluated for its activity against a cisplatin-resistant P388 subline. It showed substantial activity in this model in vitro as well as in vivo. A panel of human tumor xenografts in nude mice was treated with D-19466 or cisplatin. The effect of D-19466 was in all cases superior to that of cisplatin.
D-19466 was selected for clinical development since its pharmacological profile differs favourably from that of other platinum complexes. Phase I studies are under way and will be completed later this year.

[1] ASTA Pharma AG, D-6000 Frankfurt am Main 1
[2] Westdeutsches Tumorzentrum, D-4300 Essen 1
[3] Medizinische Universitätsklinik, D-7800 Freiburg

T 32

Overcoming cisplatin-resistance by nicotinamide in a rat ovarian tumor cell line in vitro as well as in vivo.

G. Chen and W.J. Zeller
Institute of Toxicology and Chemotherapy, German Cancer Research Center, 6900 Heidelberg, FRG

Nicotinamide (NA) significantly potentiated cytotoxicity of cisplatin (DDP) in a DDP resistant ovarian tumor cell line (O-342/DDP) in vitro, while it had no enhancing effect on DDP activity against the sensitive parental cell line (O-342), as assayed with the MTT-test. Furthermore, in vivo NA increased the mean survival time (MST) of O-342/DDP-bearing NMRI nude mice from 21.1 days in the DDP treated group to 27.1 days in the NA plus DDP treated group ($p < 0.01$), although DDP alone showed no antitumor activity in this in vivo model (MST: 20.7 days in controls vs. 21.1 in the DDP group). Activity of ADP-ribose transferase (ADPRT) was found to be almost three fold higher in O-342/DDP cells than in O-342 cells ([^{3}H]-NAD incorporation: 839 vs. 294 dpm/10^6 cells); NA (2.5 mM) inhibited ADPRT activity in O-342/DDP cells (control value: 869 dpm/10^6 cells) to a similar level (234 dpm/10^6 cells) as observed in O-342 cells without any treatment (294 dpm/10^6 cells).
Alkaline elution showed that NA had no effect on induction of DNA single strand breaks (SSB) by DDP but could significantly decrease the production of DNA interstrand crosslinks (ISCL) following DDP exposure. The latter observation parallels results obtained with 3-aminobenzamide, another inhibitor of ADPRT, which underlines that reversal of DDP-resistance must not be mediated by an increase in ISCL.

T 33

PHOTOACTIVATED NAPAVIN SUPPRESSES TUMORIGENICITY IN WILD TYPE AND MUTANT, MULTIDRUG-RESISTANT EHRLICH-LETTRÉ MOUSE ASCITES TUMOUR CELLS

C. Granzow, M. Stöhr [1], and H. Ponstingl [2]
Institut für Zell- und Tumorbiologie, [1]Institut für Radiologie und Pathophysiologie, [2]Projekt Molekulare Biologie der Mitose, Deutsches Krebsforschungszentrum, Heidelberg

The effect on tumorigenicity of Napavin, a new photoreactive vinblastine derivative (Tetrahedron Lett. 30, 5881, 1989), was investigated in the drug sensitive, wild type HD34K and in the vinblastine resistant, mutant HD33 sublines of the Ehrlich-Lettré ascites tumour. Explanted cells were incubated for 30 min with Napavin before irradiation with an argon laser (458 nm, 150 mW/cm^2, 4 min), or were left unirradiated. The Napavin concentrations used were 0.25, 0.5 and 1 µM for HD34K and 1.5, 3, and 6 µM for HD33 tumour cells. Controls were incubated with MEM, vinblastine or NAPA (the photoreactive moiety of Napavin). With the exception of the photoactivation step, all procedures involving exposure to Napavin or control substances were carried out in the dark or under light of above 650 nm wavelength. 10^5 irradiated or unirradiated test or control cells per animal were injected i.p. to groups of 5 NMRI mice each.
Two weeks later, 30/30 and 28/30 of the respective HD34K and HD33 control animals had developed large ascites tumours (>9 ml) and were sacrificed. The corresponding figures in the Napavin test groups without photoactivation were 15/15 and 14/15. However, two months later, no tumours had developed in the 15 HD33 test animals with photoactivation, whereas 2, 3, and 5 mice survived tumour-free in the HD34K test groups injected with cells incubated respectively with 0.25, 0.5, and 1 µM Napavin before irradiation.
Napavin was previously found to inhibit growth of the ascites cells in vitro transiently only (Cancer Res. 50, 403, 1990). The lasting suppression of tumorigenicity observed in vivo might be mediated immunologically after an initial growth arrest in the drug sensitive and multidrug resistant tumour cells due to activated Napavin.

T 34

SENSITIZATION OF MULTIDRUG-RESISTANT KB CELLS BY PHOTOACTIVATED NAPAVIN

C. Granzow and P. Dringsˡ
Institut für Zell- und Tumorbiologie, Deutsches Krebsforschungszentrum, Heidelberg, und ¹Thoraxklinik Heidelberg-Rohrbach

Napavin, a new photoreactive vinblastine derivative (Tetrahedron Lett. 30, 5881, 1989), sensitizes multidrug-resistant (mdr) rodent cells in vitro (Cancer Res. 50, 403, 1990), and suppresses the in vivo tumorigenicity of highly malignant drug sensitive and mdr mouse ascites cells (Granzow, Stöhr and Ponstingl, this meeting). To investigate the action of Napavin on mdr human carcinoma cells, KB cells were selected for multidrug resistance. The sublines KBC-6 and KBC-5 could be established which grow permanently with 10^{-6}M and 10^{-5}M colchicine, respectively. KBC-6 and KBC-5 cells are mdr; the respective relative resistance levels of KB, KBC-6 and KBC-5 cells are 1, 200, and 1600 for colchicine, and 1, 30 and 300 for doxorubicin. The effect of Napavin was studied using freshly harvested KB, KBC-6, and KBC-5 cells. Incubation for 30 min with Napavin was followed by resuspension in fresh medium, irradiation with an argon laser (458 nm, 0 to 15 mW/cm²) for up to 5 min, and incubation under standard conditions for a further 72 hours. Controls were exposed to culture medium, vinblastine or NAPA, the photoreactive moiety of Napavin. All steps except the photoactivation were performed in the dark or under light of above 650 nm wavelength.
The relative resistance of the cell strains to unirradiated Napavin corresponded to that determined with colchicine and doxorubicine. However, depending on resistance levels and incubation/ irradiation conditions, the photoactivation after Napavin incubation led to an increase in drug sensitivity of one to two orders of magnitude which was most prominent in KBC-5 cells and was followed by a prolonged growth arrest. These observations indicate that Napavin is a potent chemosensitizer also of mdr human carcinoma cells under conditions in vitro.
Supported by the Deutsche Krebshilfe (W 33/89/Dr 1). The authors are indebted to H. Ponstingl for the gift of Napavin and NAPA.

T 35

Reversing of multidrug resistance and persistence in murine advanced tumors by pretreatment with amphiphilic cationic drugs

H. Osswald
Institute of Toxicology and Chemotherapy, German Cancer Research Center, 6900 Heidelberg, Germany

Multidrug resistance and tumor cell persistence in advanced solid tumors represent the main problems of chemotherapy. Both phenomenons seem to be related to different alterations of the cell membrane. The mechanism of multidrug resistance is studied widely, whereas tumor cell persistence occurs predominantly in solid tumors after achieving a critical volume even if the necrobiotic part of the primary tumor and metastases increase considerably in relation to the dividing tumor cell population. The assumption of a decreased diffusion of the advanced tumor by drugs is only one insufficient explanation for the missing chemosensitivity of the tumor cells in the better perfused areas of the neoplasm. The destructive growth of the solid tumor caused a decrease of functioning lymph vessels and consequently an increase of interstitial pressure linked with a decrease of the local pH reducing not only the interstitial transport but also changing the membrane potentials in the regions as well, leading to tumor cell persistence.

Within the group of cationic amphiphilic drugs quinidine seems to be one of the interesting candidates reversing not only multidrug resistance in murine tumors (L1210, S180 Lewis Lung Carcinoma) against teniposide or mitoxantrone but also persistence in far advanced solid tumors (S180, Lewis Lung Carcinoma, M5076 Sarcoma) which are exerting a slow progression during repeated high dosed chemotherapy with cyclophosphamide, teniposide, mitoxantrone or teniposide mitoxantrone combination but can be brought to a tumor regression of more than 80 percent or to complete regression if quinidine is applied in a dose of 12-16 mg/kg sc. 1 hour before the antitumor drug(s) allowing to go beyond the previous bounds set to chemotherapy.

T 36

ANTITUMOR PROPERTIES OF OCTADECYLPHOSPHOCHOLINE (OPC); d^9-TRANS-OLEYLPHOSPHOCHOLINE (t-OlPC) AND d^9-CIS-OLEYLPHOSPHOCHOLINE (c-OlPC) IN VITRO.

S.B. Sobottka, M.R. Berger. Institute of Toxicology and Chemotherapy, German Cancer Research Center, Heidelberg, FRG.

Alkylphosphocholines (APC) are a new class of antineoplastic agents. To investigate structural requirements in the cytotoxic action of APC we examined the antiproliferative activity of three new compounds (OPC, t-OlPC and c-OlPC) in a tumor cell panel by cell count and MTT-assay.
The cell lines 1/C2 and 1/C32 (cell clones from a methylnitrosourea-induced mammary carcinoma of a female BDVI rat), MDA-MB231 and MCF-7 (human mammary carcinoma cell lines), HT-29 (human colon carcinoma) and KB (human epidermoid carcinoma) were incubated with increasing concentrations (0.4 - 250 µmol/l) of the APC derivatives up to 72 hours.
The studies revealed concentrations inhibiting cell growth by 50% (IC_{50}) ranging from 0.8 to 110 µmol/l after 72 hrs. The individual sensitivities of the cell lines were extremely different. The following table shows the IC_{50} values resulting from cell count and MTT-assay in detail:

	OPC CC/MTT	t-OlPC CC/MTT	c-OlPC CC/MTT
1/C2	21.3/22.9 µmol/l	21.8/29.3 µmol/l	41.5/ 51.0 µmol/l
1/C32	22.8/22.9 µmol/l	24.2/33.9 µmol/l	38.6/ 54.9 µmol/l
MDA-MB231	8.8/ 9.6 µmol/l	11.7/22.4 µmol/l	25.0/ 36.7 µmol/l
MCF-7	42.4/43.9 µmol/l	61.8/70.4 µmol/l	83.7/110.9 µmol/l
HT-29	4.4/ 5.9 µmol/l	5.8/ 7.1 µmol/l	17.8/ 22.1 µmol/l
KB	0.8/ 0.9 µmol/l	1.6/ 1.8 µmol/l	3.0/ 3.2 µmol/l

These data indicate that a double bond between C_9 and C_{10} within the alkyl-chain is linked with a considerable decrease of the antiproliferative activity with the cis-isomer being less effective than the trans-isomer. Following treatment with c-OlPC and t-OlPC the IC_{50} values of the MTT-assay were on average 30% higher than those from cell counting, indicating that the MTT-assay tended to underestimate the antitumor efficacy.

T 37

ANTITUMORAL ACTIVITY OF TNF, XANTHATE AND MONOCARBONIC ACID:
I. EFFECT ON HUMAN CARCINOMA XENOGRAFTS

E. Amtmann and G. Sauer
Institute for Virus Research, German Cancer Research Center, 6900 Heidelberg

The clinical use of TNF as an antitumoral agent is limited by its side effects. Here, we describe conditions, that increase the therapeutic index to such an extent that extensive necrosis can be generated in various human carcinoma xenotransplants in athymic mice after only one intravenous infusion (Amtmann and Sauer, Int. J. Cancer 45, 1113 (1990)); Sauer et al., Cancer Letters 53, 97 (1990)). These antitumoral effects are obtained in the presence of lauric acid and the xanthate compound tricyclodecan-9-yl-xanthogenate (D609) which is an inhibitor of phospholipase C (Müller-Decker, Biophys. Biochem. Res. Comm. 162, 198 (1989)) and, thus, also of protein kinase C activation (Müller-Decker et al., Exp. Cell Res. 177, 295 (1988)). The principle by which the cytocidal effect is being exerted appears to be based upon the depletion of the energy supply of tumor cells. D609, by inhibiting the activation of protein kinase C is capable of increasing the cytocidal effect of TNF, while lauric acid stimulates the synthesis of triglycerides and becomes itself incorporated. This enhanced synthesis is an energy-demanding process which in conjunction with the enhancement of the cytocidal activity of TNF at low glucose concentrations in the central parts of tumors causes cell death. Further details about the antitumoral mechanism are given in the two accompanying abstracts by Hornung et al., and Volland et al.

T 38

ANTITUMORAL ACTIVITY OF TNF, XANTHATE AND MONOCARBONIC ACID:
II. STIMULATION OF TRIACYLGLYCEROL SYNTHESIS BY MEDIUM CHAIN LENGTH MONOCARBONIC ACIDS

B. Hornung, E. Amtmann and G. Sauer
Institute for Virus Research, German Cancer Research Center, 6900 Heidelberg

The antitumoral activity of TNF is enhanced in the presence of D609 and undecanoic acid (C11) or lauric acid (C12). The synthesis of triacylglycerols was stimulated by C11 and C12 up to 10-fold in normal and tumor cell lines. Shorter or longer chain length monocarbonic acids had either no effect or were less effective. The triacylglycerol synthesis from all monocarbonic acids independent of their chain length, was found to be enhanced by C11 and C12. This leads to the speculation that C11 and C12 may possibly play a regulatory role in the synthesis of triacylglycerols. The C12-induced de novo synthesis of triacylglycerol led to a 7-fold increase of the total amount of triacylglycerols per cell during the first 10 hours of incubation. The hydrolysis of triacylglycerols was prevented by D609. Such considerable stimulation of triacylglycerol synthesis by C11 or C12 without subsequent decomposition may lead to a deficitary energy and glucose balance in the cell. Furthermore, the increased amount of C12-containing triacylglycerols, when incorporated into the cellular membrane, may alter its permeability and fluidity, thus, enhancing the cytotoxic activity of TNF.

T 39

ANTITUMORAL ACTIVITY OF TNF, XANTHATE AND MONOCARBONIC ACID:
III. THE ANTITUMORAL ACTIVITY OF TNF IS ENHANCED IN THE ABSENCE OF GLUCOSE

S. Volland, E. Amtmann and G. Sauer
Institute for Virus Research, German Cancer Research Center, 6900 Heidelberg

TNF-treated tumors in mice display extensive necrosis in their central parts while the peripheral sections are affected to a lesser extent (Sauer et al., Cancer Letters 53, 97 (1990)). In vitro most tumor cells resist the cytocidal activity of TNF. Cancer cells tend to generate their energy supply via the inefficient lactate fermentation and produce less than 1/18th of the energy that can be obtained via the aerobic pathway in normal cells. As a consequence, the glucose concentration within the tumors is reduced to about 1/100th of that in the peripheral tissue (Von Ardenne, Krebs-Mehrschritt-Therapie, Teil II (1970)). Therefore, we have examined the relationship between the glucose concentration in tissue culture medium and the cytocidal effect of TNF on the cells. Various malignant cell lines were treated with TNF in medium either with or without glucose. Increasing concentrations of (rh) TNF led to cell death in glucose-free medium, while the cell growth in glucose-containing medium remained unaffected or, was even stimulated. We found that normal fibroblasts were resistant against TNF both in the absence or presence of glucose. These results demonstrate, in compliance with the histological observations mentioned above that the antitumoral activity of TNF is enhanced not only in the central parts of tumors but also in cultures of transformed cells under conditions of limited glucose supply.

V 1

TRANSFORMATION OF MURINE FIBROBLASTS BY HERPES SIMPLEX VIRUS: LACK OF REQUIREMENT OF VIRAL DNA REPLICATION FOR TRANSFORMATION AND OF VIRAL GENE FUNCTION FOR THE STABLE MAINTENANCE OF THE TRANSFORMED STATE

G. Bauer, S. Kahl, P. Höfler, R. Gerspach, B. Matz
Abteilung Virologie, Institut für Med. Mikrobiologie und Hygiene der Universität Freiburg

The role of HSV in carcinogenesis is discussed controversely since many years. We have used a new approach to study the role of HSV in the transformation of murine fibroblasts:
i) We use an optimized transformation system which allows measurement of transformation without interference by neighbouring cells;
ii) the use of defined temperature-sensitive mutants allowed restriction to immediate early and early gene expression, thus avoiding viral DNA synthesis and virus replication. Our data show that transformation by HSV requires viral gene expression but is independent of viral DNA replication. Cells transformed by HSV maintain their state stably, i. e they show criss-cross morphology and are anchorage-independent. Daughter cells remain transformed and are free of viral DNA. These data indicate that HSV induces stable changes in the originally transformed cell which are sufficient for the maintenance and inheritance of the transformed state without further presence of viral DNA or viral gene expression.

V 2

RETROVIRUS-ONCOGENE COOPERATION ENHANCES CARCINOGENESIS IN TRANSGENIC MICE

J. Schmidt, M. Lipp, G. Brehm, A. Luz, U. Rüther, S. Wendel, P.G. Strauß, V. Erfle
GSF-Abteilung für Molekulare Zellpathologie und Institut für Pathologie, Neuherberg, EMBL Heidelberg und Ludwig-Maximilians-Universität, München

The introduction of oncogenes into the germ line of mice allows the elucidation of their specific pathogenic effects and the identification of the target tissue(s). The expression of a given oncogene in the transgenic mouse, however, is either insufficient for tumor induction or tumor incidence is generally low. This implies that oncogene expression predisposes mice to cellular alterations, and additional factors and/or consecutive events are neccessary for the generation of a progressively proliferating fully malignant cell. Endogenous retroviruses, present in numerous copies in the mouse genome and activated spontaneously or by carcinogenic agents in different mouse strains are likely candidates to act as cooperative agents in the tumorigenic process.

We have infected c-myc and c-fos transgenic mice with Akv, a replication competent prototype endogenous retrovirus. Infected mice of the two transgenic strains showed increased tumor incidence and a shortened latency period. The tumors of Akv-infected c-fos-transgenic mice differ from those which develop either in Akv-infected control mice or in c-fos transgenic mice. They contain bone together with fibrous tissue and lack invasive growth. These data indicate that endogenous retroviruses significantly enhance tumor formation in transgenic mice thereby representing a powerful model for the elucidation of their role of in carcinogenesis.

V 3

DNA-BINDING AND TRANSACTIVATION PROPERTIES OF HU-C-MYB AND V-MYB PROTEINS. S. Guehmann, A. Zobel, F. Kalkbrenner, G. Vorbrüggen and K. Moelling. Max-Planck-Institut fuer Molekulare Genetik, Abt. Schuster, Ihnestr. 73, D-1000 Berlin 33, FRG.

The human (hu-)c-Myb proto-oncogene is the cellular progenitor of the viral v-Myb oncogene and codes for a 75 kD protein involved in growth regulation and differentiation in a number of cells.

The aminoterminal DNA-binding domain of c-Myb proteins exhibits a tripartide structure with three imperfect repeats. In comparison to the proto-oncogene c-Myb the first 70 amino acid residues which contain the first half of the first repeat are deleted in v-Myb expressed in AMV. We raised monoclonal antibodies (Mab) against the c-Myb protein, one (3/7) crossreacts with v-Myb and interferes with DNA binding of c-Myb in gel retardation assays, the other one (6/22) binds to the N-terminus of c-Myb (amino acids 38-70) and increases retardation of complexes in the shift assay. This result supports the notion that the first repeat is not essential for DNA binding.

To study the function of the Myb protein as transactivator, fusion proteins were constructed in which human c-myb sequences are linked to the DNA-binding domain of the yeast transactivator GAL4. They activate transcription in transient transfection assays from a reporter gene which carries the chloramphenicol acetyl transferase (CAT) gene linked in cis to a repeat of the GAL4 binding site. Deletions of amino- and carboxyterminal sequences allowed identification of the domain responsible for transcriptional activation, which is located between amino acid residues 275 to 327. The intact GAL4-v-Myb protein can also activate transcription whereas no transactivation by the intact GAL4-c-Myb is observed, indicating that a carboxyterminal domain of c-Myb which is absent from v-Myb apparently negatively regulates transcriptional activation.

Furthermore, we noted Myb-DNA binding sites (MBS) on the upstream regulatory region of the human c-myc gene which suggested that this gene might be regulated by Myb. Footprint analyses of several regions with MBS exhibit a hierarchie of binding affinities. Cotransfection studies are underway to investigate a transactivation effect of Myb on c-myc expression at the cellular level.

V 4

DISSECTION OF TRANSFORMING AND TRANS-ACTIVATING FUNCTIONS IN THE 3'-TRANSFORMING REGION OF HUMAN PAPILLOMAVIRUS 8

T. Iftner, A.H. Müller, P.G. Fuchs and H. Pfister. Institut für Klinische und Molekulare Virologie, Loschgestr. 7, Universität Erlangen-Nürnberg, 8520 Erlangen

Human Papillomavirus (HPV) 8, which belongs to the group of HPVs associated with skin lesions, induces squamous cell carcinomas in patients with epidermodysplasia verruciformis. In a previous study we localized an in vitro transforming activity within ORF E6 but not in E7, in contrast to the genital papillomaviruses. A second transforming region could be identified in the 2.4 kb BclI-BamHI fragment covering the 3'-early domain with the trans-activating E2 ORF and ORF E4 as well as the 5'-part of ORF L2. C127 mouse fibroblast lines, containing a retroviral expression vector (pZip-Neo-SV(X)1) for the BclI-BamHI fragment, revealed a reduced serum requirement and formed colonies in soft agar but did not show any morphological alterations. To examine a possible role of the trans-activating E2 protein in the transformation process, we constructed linker insertion mutants within the E2 ORF. All cell lines established with the mutant expression vectors were severly reduced in their ability to trans-activate a HPV8 enhancer-cat-construct and could not grow in medium with low serum content. In contrast to this, a cell line containing a mutant within the carboxy terminus of E2 revealed almost wild type activity when tested for growth in soft agar. The cloning efficiencies of two other cell lines with mutations in the aminoterminal and middle part of E2 were much more reduced, but still above the level of the negative control. We are presently testing a possible participation of the E4 ORF in the anchorage independent growth, by investigating E4-specific termination mutants.

V 5

The Genesis of Harvey Sarcoma Virus: Necessary Events To Confer Transforming Function Upon Proto-*ras*

Klaus Cichutek, Iris Treinies and Matthias Lang, Paul-Ehrlich-Institute, Paul-Ehrlich-Street 51-59, D-6070 Langen, Federal Republic of Germany

The genesis of Harvey sarcoma virus (HaSV) includes insertion of a retroviral LTR into the *ras* locus, addition of a 3'LTR probably by recombination with helper virus, and viral replication of a prototype virus with proto-*ras* sequences in presence of helper virus. Further structural changes in the course of the generation of HaSV from such a prototype virus were defined as (1) deletion of the cellular *ras* promotor and poly(a)-signal, (2) deletion of all or parts of non-coding exons, one of them exon 1 (Cichutek et al., PNAS 83, 2340, 1986; Cichutek et al., J.Virol. 63, 1377, 1990), (3) introduction of virus-specific codons 12 and 59 by point mutation. To test the oncogenic potential of *ras* genes that include some of the described structural changes, we constructed various normal Harvey *ras* genes with LTRs inserted in the vicinity including a prototype viral *ras* gene. Transfection into NIH 3T3 cells demonstrated that 5'but not 3'LTR-insertion is sufficient to activate the transforming potential of proto-*ras* in cell lines. LTR-insertions into normal proto-*ras* have been found in a chicken tumor and a tumorigenic bone marrow cell line and can thus be correlated with the subsequent malignant transformation.

LTR-*ras* genes were unable to elicit transformation of primary rat embryo fibroblasts (REF) after transfection, whereas replicating prototype viruses with proto-*ras* codons and cellular promotor elements efficiently transformed REF. In addition, we were able to demonstrate a strong upregulation of proto-*jun* mRNA expression in such cells. The contribution of this effect to transformation is currently under investigation. To test the hypothesis that viral replication is necesary for primary cell but not cell line transformation, we generated prototype Harvey sarcoma virus variants in helperfree cells the transformation potentials of which are currently tested. In addition, a new homology region 5'of exon1 of proto-*ras* that is also found in the viral *ras* sequence is functionally tested to further investigate so far unknown elements of normal proto-*ras*.

V 6

AN SV40-T-ANTIGEN-SPECIFIC ANTIBODY, PAB 1605, REACTIVE WITH A HUMAN LYMPHOMA PROTEIN

C. Braun, C. Haessler, M. Fritsche, and G. Brandner, Abt. Virologie, Inst. f. Medizinische Mikrobiologie & Hygiene der Univ., 78 Freiburg.

PAb 1605, a monoclonal antibody directed against Simian Virus 40 (SV40) large tumor(T) antigen is specific for the sequence thr(701)-pro(706) within the C-terminal undecapeptide, lys(698)-thr(708), of this protein [EMBO J. 3: 1485-1491 (1984); Virology 166: 245-247 (1988)]. The same sequence is an epitope also for cytotoxic lymphocytes directed against SV40-transformed mouse cells [J. Cancer Res. Clin. Oncol. 115: S44 (1989)].
However, PAb 1605 is reactive also with human SV40-negative lymphoid cells: We have found an antigen expressed in B- and T-lymphoma cells, but not in untransformed peripheral blood lymphocytes. Upon cell extraction, separation in SDS-polyacrylamide gels and immunoblotting, a protein becomes visible with PAb 1605 exhibiting an apparent molecular weight of approx. 30 kD.
The sequence homology of this unknown human lymphoma protein with SV40 T-antigen is probably low since the monoclonal antibody KT3, specific for the adjacent sequence pro(703)-thr(708) within the C-terminal undecapeptide of SV40 T-antigen [H. MacArthur, G. Walter, J. Virol. 51: 670-681 (1984)] gave no positive immunofluorescence with several human B-lymphoma cells. In addition, a protein sequence data search revealed no homology of lys(698)-thr(708) or other SV40 T-antigen sequence with known human lymphoid proteins. Thus, the molecular basis of this mimicry remains unknown as yet.
(Supported by BMFT, 01GA8709, Stiftung Müller-Fahnenberg, and DFG, Br 281/11.2).

V 7

A SYNTHETIC PEPTIDE DERIVED FROM THE ENVELOPE PROTEIN gp41 of HIV-1 SUPPRESSES LYMPHOCYTE PROLIFERATION

Denner, J., Norley, S., and Kurth, R.
Paul-Ehrlich-Institut, Langen

Although the genome of HIV-1 is well studied, the sequence(s) responsible for its immunopathogenicity is still unknown. We provide evidence that a 17-mer peptide, derived from the transmembrane glycoprotein gp41 of HIV-1 (aa583-599) inhibits Con A-induced stimulation of human lymphocytes. This peptide is related to the evolutionary highly conserved immunosuppressive peptides of type C and D retroviruses. Like these it is interspecies-active, e.g. it inhibits IL-2 dependent proliferation of murine T-cells. In order to suppress the *in vitro* immune reactions the peptide has to be conjugated to a carrier protein, so simulating an intact gp41 molecule. Unconjugated peptide and control peptide conjugates have no inhibitory effect. The N-terminal octamer (aa583-590) represents the minimal immunosuppressive domain and there is an immunogenic epitope located C-terminal to this region. The humoral immune response of responding AIDS patients decreases with progression of the disease. Although the immunosuppressive activity of this peptide is clearly shown, its mechanism of action and its possible role in AIDS is still unclear. However it has been shown that during progression of AIDS the titer of HIV-1 and therefore the concentration of the immunosuppressive peptide increases. Using immunoflurescence methods and biotinylated peptide we are now characterising the putative receptor on the cell membrane of lymphocytes.
Comparison of the immunosuppressive peptide of HIV-1 with corresponding sequences of less pathogenic human (HIV-2) and non-pathogenic simian immunodeficiency viruses (SIVagm) revealed considerable differences in their amino acid sequences and their immunosuppressive activities.
In addition, the immunosuppressive peptide of HIV-1 inhibits the cytopathic effect of HIV-1 on MT4 cells, suggesting that it may interfere with virus infection. Again, only conjugated HIV583-599 has this property, whereas unconjugated peptide and control peptide conjugates are ineffective.

V 8

INHIBITION OF TRANSCRIPTION OF THE T-CELL LYMPHOMA INDUCING MOLONEY-MURINE-LEUKEMIA VIRUS BY A XANTHATE COMPOUND

L. Music and G. Sauer
Institute for Virus Research, German Cancer Research Center, 6900 Heidelberg

We are interested in the mechanism controlling the expression of proviral moloney-murine-leukemia virus (Mo-MLV) genomes. In particular, the regulation of viral transcription was investigated by using stably transformed Mo-MLV NIH-mouse fibroblasts. After treatment of these producer cells for 24 h with the antiviral xanthate D609 the production of infectious virus was inhibited up to 90 %. This effect was accompanied by the selective inhibition of viral transcripts as shown in Northern blots. Additionally, immunoblot assays demonstrated the lack of viral proteins after treatment of producer cells with D609. The compound inhibits the activation of some phospholipase C isoenzymes and blocks as a consequence the protein kinase C mediated phosphorylation of different target proteins amongst which transacting factors could be identified. The functional significance of such modification of transacting factors that control the viral transcription is examined.

Z 1

ONCOGENE COMPLEMENTATION IN FETAL BRAIN TRANSPLANTS

O.D. Wiestler, A. Aguzzi, A. von Deimling, M. Schneemann, R. Eibl, P. Kleihues and H. Land
Abteilung Neuropathologie, Universitätsspital Zürich und Imperial Cancer Research Fund, London

Using a neural transplantation model and retrovirus-mediated gene transfer, we have introduced activated oncogenes into the developing rat brain. With mock-infected donor cells, the transplants gave rise to highly differentiated neuroectodermal tissue containing all major CNS cell types. This neural transplantation system was used to study potential effects of the oncogenes v-Ha-*ras* and v-*myc* on the developing central nervous system *in vivo*. Upon insertion of a construct encoding v-Ha-*ras* and the E. coli ß-galactosidase marker gene, the retroviral vector was found to be expressed in neurons, astrocytes and endothelial cells of the graft. After latency periods of several months, immature glial neoplasms were observed in 50 % of the transplants exposed to this retroviral vector. By X-gal histochemistry, the retroviral construct was shown to be highly expressed in the tumors as well as in morphologically intact donor cells indicating that an activated *Ha-ras* oncogene has the potential to initiate neoplastic transformation of glial cells. Introduction of the v-*myc* oncogene into 15 grafts resulted in only a single primitive neuroectodermal tumor. Preliminary evidence suggests that this neoplam resulted from insertional mutagenesis of a cellular gene. A replication-defective retroviral vector which encodes both oncogenes was used to study a complementary effect of these transforming genes on the brain. Simultaneous expression of the v-Ha-*ras* and v-*myc* genes yielded highly malignant, polyclonal neoplasms in all recipient animals with vital grafts. Large tumors were observed as early as 13 days after transplantation. In addition, neoplastic transformation was also observed *in vitro* following introduction of the *ras&myc* construct into embryonic CNS cultures. These data indicate that *ras* and *myc* oncogenes exert a powerful complementary transformation potential on neural progenitor cells both, *in vitro* and *in vivo*.

Z 2

Induction of Differentiation in Choriocarcinoma Cells by Extracellular Matrix: On the Role of Physical Properties of the Matrix.

H.-P. Hohn*, M. Höök*, H.-W. Denker*;

*) Inst. f. Anatomie, University Hospital, Essen, FRG;
*) Dept. of Biochemistry, University of Alabama at Birmingham, USA

Human choriocarcinoma cells provide a useful experimental model for the study of differentiation potentials of tumor cells and their role in malignancy. We have investigated the ability of extracellular matrix to induce differentiation in BeWo choriocarcinoma cells in vitro. The response of all differentiation markers monitored (steroid and peptide hormone production, proliferation, morphology, alkaline phosphatase) was highly pronounced on three-dimensional flexible matrices (gels: collagen I, Matrigel™, placental matrix) as opposed to rigid substrata (including additionally: fibronectin, laminin, collagen IV). The results indicate that not only the type of effector molecules but also the physical properties of the matrix contribute important information for the induction of differentiation in trophoblast tumor cells. This is supported by experiments with artificial flexible substrata (gels of derivatized agarose with or without matrix molecules coupled to it) or on plastic coated with poly-HEMA (different concentrations: modulation of cell shape). Cells always showed increased expression of differentiation markers when having a rounded as opposed to flattened morphology. It is concluded that modification of cell shape can provide a differentiation stimulus for choriocarcinoma cells that does not necessarily depend on the recognition of specific adhesion molecules.

Supported by Dr. Mildred Scheel Stiftung für Krebsforschung

Z 3

Rapid aggregation and tight junction formation in single cell suspensions of tumor cells after very low dose trypsin treatment

W.Kemmner and K.Zaar, Inst. f. Biochemie II and Inst. f. Anatomie und Zellbiologie II, Universität Heidelberg.

The tight junction (zonula occludens), a belt-like region of contact on the apical pole of epithelial cells, serves as a barrier controlling the paracellular pathway of large molecules. A variety of experimental conditions have been reported to induce tight junctions in cultured cells or tissues. The human adenocarcinoma cell HT29 in culture virtually grows without tight junctions. After short trypsin treatment of adherent confluent cells grown on petri-dishes for 4 to 5 days the formation of tight junctions on 50-60% of plasma membranes counted on freeze-fracture replicas has been found. In order to further study the relationship between tight junction formation and intercellular adhesion of tumor cells we have examined the trypsin-mediated processes in suspensions of single tumor cells after their detachment from culture dishes. In such a system both aggregation and tight junction formation can be followed. Very low dose trypsin treatment (0.15 µg/ml,30 min, 37°C) induced rapid intercellular adhesion of suspended HT29 colon carcinoma cells. Intercellular adhesion was independent of the presence of divalent cations. Electron microscopy of freeze-fractured membrane fragments of trypsin-treated HT29 cells revealed a dramatic increase of typical tight junction structures during aggregation.

Z 4

Influence of MyoD1 and 5-aza-2'deoxycytidine treatment on the stability of differentiation of the human skin kertinocytes line HaCaT

Petra Boukamp*, Peter A. Jones**. and Norbert E. Fusenig*; * DKFZ, Heidelberg, FRG; ** USC, Los Angeles, USA

Epidermal differentiation is a complex process strictly linked to the structural organization of the stratified squamous epithelium. This process involves an ordered sequence of defined morphological changes, accompanied by the sequential expression and modification of specific differentiation products. While many of these products are well characterized little is known about the mechanism or genes involved in the regulation of the differentiation process. For myogenesis, on the other hand, regulatory genes are described with the propensity to induce myogenic differentiation also in non-muscle cells. One of these genes MyoD1 (myogenic determination gene 1) was transfected into the spontaneously immortalized human keratinocyte line HaCaT and the consequence of the forced expression of MyoD1 in a cell of ectodermal linage analyzed, as to whether the myogenic expression would affect growth and differentiation properties of the well characterized epidermal phenotype. MyoD1 expression in the HaCaT cells resulted in the induction of myogenic differentiation products (myosin, desmin, vimentin) without disturbing epidermal differentiation in vitro (keratin profile) and in vivo (formation of a well stratified and differentiated epidermis in transplants). Additional treatment with the hypomethylating drug 5-aza-CdR led to loss of tissue morphogenesis without affecting the expression of cyto-differentiation makers, indicating two independent modes of regulation. From these cells a subfraction could be isolated which no longer expressed any of the epidermal differentiation markers neither in vitro nor in vivo but instead morphological and biochemical traits of mesenchymal /myogenic cells.

Thus, by introducing a muscle determination gene into human epidermal cells and by decreasing the methylation status (treatment with 5-aza-CdR) we could induce a shift from epidermal to mesenchymal/myogenic phenotype, indicating that fully determined epidermal cells are able to "trans-differentiate" under certain conditions. On the other hand, the data also stress the tenacity with which the epidermal phenotype is maintained despite major manipulations.

Z 5

DEVELOPMENT OF INVASIVE AND GROWTH FACTOR-INDEPENDENT PRIMARY HUMAN MELANOMAS

R. Kath und M. Herlyn*

Universitätsklinikum Essen, Innere Klinik und Poliklinik (Tumorforschung), *Wistar Institute of Anatomy and Biology, Philadelphia, PA

Growth autonomy and high levels of invasiveness are characteristics of human melanoma cell lines that are metastatic in vivo. By consecutive passage through a reconstructed basement membrane, we have selected from 5 out of 6 primary melanoma cell lines variants which show an up to 10-fold increase in invasiveness. The invasive variants grew more rapidly than the parental, non-invasive cells in serum- and growth factor-free medium and one of the three variant cell lines with the highest invasive capacity in vitro metastasized to the lungs when injected subcutaneously into nude mice. In a second approach, variants of six primary melanoma cell lines were clonally selected in medium without exogenous growth factors (protein-free medium). These selected cells showed higher invasive properties in vitro and in vivo than the parental cells. Clones of invasive and growth factor-independent cell variants were heterogenous and changed over time in the absence of selected pressure to a phenotype similar to that of parental non-selected cells. These results indicate that primary melanoma cells contain subpopulations of cells that have the phenotype of an advanced (metastatic) stage of tumor progression, but this phenotype is not stable without selective pressure.

Z 6

INDENTIFICATION OF NONGENOTOXIC CARCINOGENS IN HEPATOCYTE CULTURES BY FLOW CYTOMETRY

P.Maier, H.P.Schawalder, J.Elsner. Inst. für Toxikologie, Universität und ETH Zürich

The response of the nongenotoxic rodent carcinogen thioacetamide (TA) was characterized in rat hepatocytes cultured at 4% O_2 or 13% O_2. Cells were exposed to TA from 24 -72 hours after isolation at exposure levels between 0.01 and 0.33mM. Hepatocytes and isolated nuclei of them were analyzed by DNA-protein flow cytometry. The proportion of S-phase cells and of diploid, tetraploid or octoploid as well as of binucleated hepatocytes were measured or calculated. The proportion of S-phase cells in diploid hepatocytes increased with increasing concentration of TA up to 3.9 fold. The corresponding increase in S-phase mononucleated tetraploid cells was only 1.8 fold and S-phase binucleated tetraploid cells showed no increase. The mitogenic stimuli was stronger in cultures maintained at 4% O_2. The relative contribution of binuclear cells was increased 1.2-fold in the tetraploid and 1.5-fold in the octoploid hepatocytes. Therefore TA stimulates preferentially diploid cells to undergo complete mitosis and/or post mitotic diploid binucleated cells to undergo cytokinesis. The mitogenic activity of the chemical was not associated with an increase in cellular protein content. The results obtained correspond well with data from in vivo studies.

Z 7

ACTION OF RAT LIVER TUMOR PROMOTERS ON DNA SYNTHESIS IN CULTURED HUMAN HEPATOCYTES.

W.Parzefall, E.Erber, R.Sedivy, R.Schulte-Hermann
Inst. für Tumorbiologie-Krebsforschung,
Borschkegasse 8a, A-1090 Wien, Austria

A large number of compounds in divergent human use are known to accelerate and enhance liver tumor formation in rodents after the animals have been dosed by an initiating carcinogen. This applies e.g. to the following model compounds: cyproterone acetate (CPA), α-hexachlorocyclohexane, (HCH); Pregnenolon-16α-carbonitril, (PCN); Nafenopin, (NAF), and Phenobarbital, (PB). These so-called tumor-promoters are also inducers of drug metabolizing enzymes and of liver growth in intact rats. It is not known whether or not they pose a risk for human cancer formation. The growth response is thought to be a common property of liver tumor promoters. Therefore we have developed a culture system with primary rat hepatocytes to test for the induction of DNA synthesis by measuring thymidine incorporation either biochemically or by autoradiographic techniques as an indicator of possible tumor promoting activity. The growth factor EGF served as reference stimulus. It was found that HCH, PCN and NAF stimulated DNA synthesis in a dose dependent manner after a lag phase of approximately 24 hours. PB was the least potent of all compounds. EGF was consistently stimulatory in rat cells. The very culture conditions were then used with seven preparations of human hepatocytes. Again, EGF induced DNA synthesis, although the number of labeled cells was approximately by an order of magnitude lower than with rat hepatocytes. In contrast to rat hepatocytes no significant increases in DNA synthesis could be found after treatment with CPA, HCH, NAF, PB or with rifampicin. These findings suggest that human hepatocytes are much less sensitive to growth stimulation by rat liver tumor promoters and possibly also to their tumorigenic effects. A larger number of preparations will be needed to prove the universality of these findings.

Z 8

DNA PLOIDY AND METASTASIS IN ORAL CARCINOMA

J. Hemmer
Dept. of Maxillofacial Surgery, Laboratory for Experimental Cytology, University of Ulm, FRG.

The ploidy status of 183 primary squamous cell carcinomas of the oral cavity was analysed by DNA flow cytometry. In 73% of the cases, cell lines with abnormal DNA content were detected. Aneuploidy rate increased with size from 21% in T1 to 76% in T2 and 91% in T3. Obviously, early tumor stages consist of diploid tumor cells only, the emergence of aneuploid cell lines represents a characteristic property of late tumor progression. Furthermore, 88% of the patients with cervical lymph node involvement had aneuploid primary tumors. Only a minority of diploid cases had metastases indicating a largely reduced ability of cell dissemination of diploid clones. Cell lines of corresponding metastases and primary tumors showed identical DNA contents indicating that the aneuploid genotype, in fact, represents a highly metastatic phenotype.

Z 9

Reserved differentiation capacity of SV40, HPV and spontaneous immortalized human keratinocytes.

P.Tomakidi, P.Boukamp, D.Breitkreutz, N.E.Fusenig
DKFZ-Institut für Biochemie, Heidelberg.

From earlier studies it had been postulated that block in differentiation is an early and obligatory prerequisite for immortalization of human skin keratinocytes. To test this hypothesis we have investigated the differentiation capacity in vitro and in vivo of six human keratinocyte cell lines which were either obtained after transfection with SV40 (HaSV, SVK14) or HPV16 (HPKI and II), or arose spontaneously (HaCaT, NM1). Under in vitro conditions all lines expressed the keratin typical for epidermal basal cells (K14), the "hyperproliferative" keratins K6 and K16 as well as the "simple type" keratins K7, 8, 18 and 19. The differentiation specific keratins K1 and 10 were expressed by the spontaneously immortalized as well as HPV16 transfected cell lines but absent in the SV-40 immortialized cell lines. These data obtained by 2D gelelectrophoresis were confirmed by immunofluorescence using specific antibodies for the respective keratins. In vivo differentiation was tested after transplantation of the cells as organotypic cultures (on collagen gels) onto nude mice. All cell lines developed stratified epidermis-like epithelia except NM1 and SVK14. Moreover, the simple type keratins were largely down-regulated or completely suppressed in the transplants and the epidermal keratins became predominant, indicating that these cell lines had maintained the propensity for epidermal differentiation upon appropriate stimulation. On the other hand SVK14 formed undifferentiated epithelia with no indication of differentiation specific keratins, while NM1 did not stratify well but synthesised K1 and 10. This clearly demonstrates that, with one exception, (i) all cell lines are still capable to express epidermal differentiation markers although to a variable degree and (ii) the degree of differentiation did not correlate with the respective mode of immortalization.

Z 10

Perfusion and metabolic micromilieu of tumors derived from ras transformed fibroblasts

F. Kallinowski, W. Strauss[1], A.L. Brownell[2]
Chirurg. Univ.-Klinik Heidelberg, [1]Dept. of Nuclear Medicine, and [2]Physics Research Laboratory, Massachusetts General Hospital, Boston, USA

Mutated ras genes are frequently found in human tumors. Ras transformed fibroblasts in nude mice were studied in order to assess therapeutically relevant changes of the tumor micromilieu. Tissue perfusion was obtained with a radioactive tracer technique using thallous chloride. Tumor tissue oxygenation was evaluated with oxygen sensitive electrodes. Glucose utilization was imaged with high resolution positron emission tomography after the injection of ^{18}F-2-deoxy-D-glucose.

Results: Compared with parental Rat1 cells, ras transformed tumors exhibited accelerated growth rates. Perfusion rates of ras transformed tumor lines were reduced (mean ± SE: 0.68 ± 0.12 vs. 0.44 ± 0.08 ml/g/min). This was concomitant with a reduction in median oxygenation values from 14 to 8 mmHg. In contrast, similar glucose metabolic rates were found in all tumor lines (11.0 ± 2.1 vs. 11.6 ± 1.7 µmol/100cc/min).

It is concluded that ras transformation accelerates growth leading to a reduction of tumor perfusion and oxygenation. An increased glucose extraction compensates the reduced supply leading to comparable metabolic rates.

Z 11

PRESERVATION AND REUTILIZATION OF THE VASCULAR TUMOR BED DURING REPOPULATION OF THE MOUSE ADENOCARCINOMA EO771 AFTER CYCLOPHOSPHAMIDE

I.D. Bassukas, G. Vester and B. Maurer-Schultze
Inst. f. Med. Strahlenkunde d. Universität Würzburg

Tumor growth depends on anglogenesis, however, how far this is also true for tumor repopulation after treatment is not known. Kinetics of tumor cell repopulation and simultaneously of intratumoral endothelial cell proliferation of the transplantable murine adenocarcinoma EO771 have been studied after a single injection of cyclophosphamide (CY; 300 mg/kg). Tumor size, cell number/tumor, tumor cell size, DNA histograms and ^{3}H-thymidine (TdR) labeling indices of tumor cells (LItu) and of endothelial cells (LIend) have been determined as a function of time after tumor inoculation and treatment using morphometry, DNA flow cytometry and ^{3}H-TdR autoradiography.
The untreated tumor grows according to the Gompertz mode of growth with LI of tumor and endothelial cells decreasing as a function of tumor age. Although almost all tumor cells are killed by one single injection of CY (log kill~6), there is no corresponding reduction in tumor size, since some sublethally damaged tumor cells (~15%) develop via multiple endomitoses into giant reproductive sterile cells which fill the place of the tumor cells that are immediately killed and removed (~85%). About one week after treatment tumor repopulation starts with aggregates of typical tumor cells in the close vicinity of preserved, sinusoid-like enlarged intratumoral capillaries. Two weeks later these cells have spread throughout the entire tumor by infiltrating and destructing the giant tumor cells. This process is accompagnied by a sharp increase of the LItu up to values higher than that at the time of treatment. In contrast to the LItu the LIend does not increase during tumor cell repopulation.
These findings suggest that the repopulation of the tumor after treatment does not depend on angiogenesis, however, the regrowing tumor reutilizes the preserved vascular bed of the tumor which is protected from collapsing by the giant tumor cells providing the rapid tumor relapse after subcurative treatment.

Z 12

CHEMICALLY-INDUCED MURINE RHABDOMYOSARCOMA (RMS) - TUMORBIOLOGICAL ASPECTS OF SARCOMAS, CELL CULTURES AND NUDE MOUSE ALLOTRANSPLANTS.

L. Langbein, H. Kosmehl, D. Katenkamp.

Institute of Cell- and Tumorbiology, DKFZ, Heidelberg; Institute of Pathology, Friedrich Schiller University, Jena

Human and experimental RMSs comprise a mixture of primitive mesenchymal cells and cells showing various stages of rhabdomyomatous differentiation (RHD). The qualitative and quantitative degree of RHD can be evaluated by morphology (light- and electron microscopy) and expression of defined structural and functional proteins (vimentin, desmin, cytokeratins No. 8, 18, 19; actin, myoglobin, fibronectin, laminin) which were considered to give informations on tumor diagnosis as well as prognosis and biological behavior of RMS. We investigated aspects which may influence RHD and cellular maturation in Methylcholanthrene-induced murine RMS in vivo, including after surgical tumor diminution, in differentiated cell cultures (CC) with varying growth conditions and after (re-)transplantation (tumor pieces, cultured cells) into athymic nude mice. When we compare solid tumors with adherent CC of decreasing cell densities -up to single cells- with suspension CC, a gradual decline of RHD ("dedifferentiation") was seen paralleled by a decrease or lost of markers (e.g. desmin, myoglobin; cytokeratins only in tumor tissue) and clearly correlatable to a lowered extent of cell-cell/cell-substrate contacts and changed extracellular matrix formations. In suspension CC without any cellular contacts, no RHD was seen. The process of dedifferentiation was reversible after restoration of cell contacts (adherent growth with increasing cell densities) or after transplantation. Therefore we suggest that the extent of cell-substrate contacts and number of cell-cell interactions, both mediated by extracellular matrix components, are responsible, in part at least, for the capability of a malignant mesenchymal cell, which is able to undergo RHD, to achieve various stages of maturation. Transplantation of RMS (cells from cultures or tissue pieces) results in differentiated or dedifferentiated solid tumor allografts independent on the RHD of original tumors. Metastases in original tumors were seen very rare, even after surgical diminution of RMS. Only after transplantation of dedifferentiated cultured RMS (appr. 80. Pass) followed by tumor surgery metastatic rate was significantly enhanced suggesting dedifferentiation as a possible prerequisite of metastazation in RMS.

Z 13

CATHEPSIN B: ACTIVITY AND CYTOCHEMISTRY IN HUMAN LUNG TUMOR CELL LINES

EBERHARD SPIESS[1], GÜNTHER TREFZ[2], and WERNER EBERT[2]
[1]Institut für Zell und Tumorbiologie, DKFZ, D 6900 Heidelberg, FRG und [2]Thoraxklinik der LVA Baden, D 6900 Heidelberg, FRG.

Proteolytic activity is considered to play an essential role in the invasive process which is necessarily envolved in the metastatic cascade. The cystein proteinase cathepsin B (CB) has attrackted particular interest as CB-like activities appeared abnormally also outside the lysosomal compartment in tumors or tumor cells.
We have investigated human lung tumor derived cell lines (HS24 isolated from a squamous cell carcinoma, SB3 isolated from a metastasis of an adenocarcinoma) for CB activities by biochemical and cytochemical methods using synthetic substrates (Z-Arg-Arg-AMC, Z-Arg-Arg-4MβNA, Z-Val-Lys-Lys-Arg-4MβNA).
Both cell lines are potent CB producers. Under normal culture conditions neither active nor latent CB is secreted into the culture medium. CB release could be stimulated by the addition of EGF and NaCl at concentrations of 10 ng/ml and 150 mM, respectively. In contrast to purified human CB (pH optimum at 6.2) the pH optimum of CB activity shifted in extracts of tumor cells to 6.8 - 7.2; however, a similar result was found for the same type of extract from normal human fibroblasts (Wi38). On the cellular level in living or formaldehyde fixed cells, the CB activity peaked at pH 6.2, was almost unreduced at pH 6.8 and was still considerably high at pH 7.2 in all mentioned cells. Cytochemical localization at the light microscopical level revealed intracellular but also plasmamembrane associated activity. These results were confirmed at the electron microscopical level: besides lysosomes, the ER showed high activity; but also the nuclear membrane and the plasmamembrane showed centers of activity.
These experiments reveal the first indication for a possible involvement of EGF in the release of CB, show CB activity at non-lysosomal pH values under in vivo conditions and directly prove the non-lysosomal localization of CB in tumor cells confirming biochemical data from the literature. The results thus open the possibility of an involvement of released or plasmamembrane bound CB in the degradation (direct or indirect) of extracellular matrix material and a potential role of this enzyme in the course of the invasive and metastatic process.

Z 14

CELL-SURFACE PHOSVITIN/CASEIN KINASE:
ASPECTS OF SUBSTRATE/ENZYME ORIENTATION ON THE MEMBRANE

Peter Jordan and Dieter Kübler
Department of Pathochemistry, German Cancer Research Center, Heidelberg

A number of cell types carry two kinds of protein kinase (PK) activity on their extracellular surface (ectoPK) with characteristics of phosvitin/casein type II-PK (JBC 257, 322) and cAMP-dependent PK (JBC 264, 14599). The casein kinase catalyses phosphorylation of a pattern of plasmamembrane proteins characteristic for each cell type as well as exogenous substrate proteins. Two highly phosphorylated surface proteins, designated pp 120 and pp 100, are wide-spread among cells and have been characterized previously as integral membrane proteins. In this study we have attempted to clarify the spatial relationship of these two proteins by investigating their accessibility to externally applied modifiers. Membrane-impermeable cross-linking agents of the succinimidyl-type (DSP and SAND) affected some of the surface phosphoproteins, but pp 120 and pp 100 were cross-linked neither with each other nor to other membrane proteins. The pp 120-phosphorylation alone was abolished in the presence of 2 mM Ca^{2+}. This led us to look at several other divalent cations. The results, showing inhibition by different cations of pp 120-phosphorylation, suggest a conformational change of the protein in the membrane.
Exogenous substrate proteins can release ectoPK from the intact cell into the incubation fluid (PNAS 80, 4021) by a yet unknown mechanism. Thus, membrane protein phosphorylation might be controlled by the available ectoPK activity. In view of the fact that several other ectoenzymes are membrane-bound via a glycosyl-phosphatidylinositol anchor we looked for such linkage of ectoPK. Therefore, HeLa cells were incubated with PI-PLC: however, no ectoPK activity was liberated by this treatment.

Z 15

EPIDERMAL GROWTH FACTOR RECEPTORS EXPRESSED ON METASTATIC 13762NF RAT MAMMARY ADENOCARCINOMA CELLS EXHIBIT LIGAND INDUCED ACTIVATION WITHOUT SIGNIFICANT RECEPTOR PHOSPHORYLATION

Rosemarie B. Lichtner[1], Marion Wiedemuth[1], Annette Kittmann[1], Axel Ullrich[2], Volker Schirrmacher[1] ad Khashayarsha Khazaie[1]
[1]Department of Immunology and Genetics, German Cancer Research Center, 6900 Heidelberg and [2]Max Planck Institute for Biochemistry, 8033 Martinsried, Federal Republic of Germany.

Expression and function of epidermal growth factor receptor (EGFR) was investigated in a metastatic cell clone (MTLn3) derived from the 13762NF rat mammary adenocarcinoma. MTLn3 cells showed growth in response to EGF and expressed EGFR specific transcripts. Cross-linking experiments with ^{125}I-EGF, as well as ^{35}S-methionine labelling followed by immunoprecipitation with receptor specific antibodies readily detected in MTLn3 cells the expected 170 kd EGFR protein. However, no receptor phosphorylation could be identified in intact cells or in membrane preparations, while EGF-dependent phosphorylation of substrates occured in intact cells. Furthermore, EGFRs bound in immunocomplexes or associated with the cytoskeleton of detergent treated cells were able to undergo basal and EGF-induced phosphorylation in vitro. In addition, two proteins with M_r of 420-480 and 95 kd specifically bound ^{125}I-EGF on intact MTLn3 and sparse cultures of A431 cells. Only the 420-480 but not the 95 kd protein was phosphorylated. The functional activity of EGFR without detectable receptor autophosphorylation and the presence of alternative forms of EGFR in the highly metastatic cell clone MTLn3 were unexpected. These novel properties of MTLn3 may possibly relate to the aberrant growth and dissemination of these cells in syngeneic animals.

Z 16

ON THE ROLE OF THE NEURAL CELL ADHESION MOLECULE L1 IN METASTASIS OF THE MURINE LYMPHOMA ADHESION VARIANT ESB-MP.

Aiga Kowitz, Guni Kadmon, Volker Schirrmacher, and Peter Altevogt
Institut für Immunologie und Genetik, Deutsches Krebsforschungszentrum, 6900 Heidelberg, F.R.G.

Cell adhesion molecules may be involved in a variety of cellular functions and may play a role in certain stages of the metastatic process of tumor cells.
We have studied an adhesive tumor cell variant (ESb-MP) of the highly metastatic murine lymphoma ESb. In contrast to the parental cells the adhesive variant ESb-MP is decreased in malignancy and had aquired an increased expression of several adhesion molecules including the neural adhesion molecule L1 and the cell-cell interaction molecules CD2 and LFA-1.
In further experiments we have investigated the influence of L1 expression on malignant behaviour of ESb-MP lymphoid cells. First, L1-negative variants of these cells were established using fluorescence-activated cell sorting. Second, L1-positive revertants of the L1-negative variants were selected using the same proceedure. Testing the malignancy of these different L1 expressing clones in syngenic mice revealed that L1-negative clones lead to enhanced metastasis and earlier death of the animals.
Since L1 mediates Ca^{2+}-dependent cell-cell interaction the homotypic aggregation of these ESb-MP clones was studied in Ca^{2+}-low medium. It could be shown that the L1-dependent aggregation was their only Ca^{2+}-independent mechanism. The L1-dependent homotypic aggregation of these different L1-expressing clones in vitro was found to be correlated with their malignancy in vivo.
This suggests that L1 mediated cell-cell adhesion at the site of the primary tumor and/or metastases would lead to reduced release of tumor cells.

Z 17

E-CADHERIN-MEDIATED CELL-CELL ADHESION PREVENTS INVASIVENESS OF HUMAN CARCINOMA CELLS

U.H. Frixen, J. Behrens, J. Schipper, M. Sachs, and W. Birchmeier
Institut für Zellbiologie (Tumorforschung), Essen Medical School, Virchowstr. 173, 4300 Essen 1, FRG

The ability of carcinomas to invade and to metastasize largely depends on the degree of epithelial differentiation within the tumors, i.e. poorly differentiated being more invasive than well differentiated carcinomas. Here we confirmed this correlation by examining various human cell lines derived from bladder, breast, lung, and pancreas carcinomas. We found that carcinoma cell lines with an epithelioid phenotype were noninvasive and expressed the epithelium-specific cell-cell adhesion molecule E-cadherin (also known as Arc-1, uvomorulin, and cell-CAM 120/80), as visualized by immunofluorescence microscopy and by Western and Northern blotting, whereas carcinoma cell lines with a fibroblastoid phenotype were invasive and had lost E-cadherin expression. Invasiveness of these latter cell could be prevented by transfection with E-cadherin cDNA and was again induced by treatment of the transfected cells with anti-E-cadherin monoclonal antibodies. The analysis of normal human squamous epithelium and 18 squamous cell carcinomas of the head and neck revealed that E-cadherin is expressed in normal tissue and well differentiated tumors (6), weakly expressed in moderately (7) and not present in poorly differentiated tumors (5). These findings indicate that the selective loss of E-cadherin expression can generate dedifferentiation and invasiveness of human carcinoma cells, and they suggest further that E-cadherin acts as an invasion suppressor.

Z 18

AUTOREGULATION OF ANDROGEN RECEPTOR LEVEL IN THE NEW HUMAN BREAST CANCER CELL LINE MFM-223

R. Hackenberg, Th. Hawighorst, J. Hofmann, M. Beato* and K.-D. Schulz
Frauenklinik und Institut für Molekularbiologie und Tumorforschung* der Philipps-Universität Marburg

The recently established cell line MFM-223 contains high levels of androgen receptors (90.000 binding sites per cell, K_d = 0.12 nM), but lacks significant amounts of estrogen and progesterone receptors. Proliferation is effectively inhibited by 10 nM DHT. Inhibition by 10 nM DHT can be partially antagonized by antiandrogens underlining the involvement of the androgen receptor.

AR protein level was assayed by a whole cell binding assay using ^{3}H-R1881. After preincubation with androgen, cells were rinsed severall times prior to the receptor assay. Complete removal of the unlabeld androgen was confirmed by constant K_d-values. AR level was down regulated to 60% after treatment with 10 nM DHT for 24h. Incubation for 14 days yielded a down regulation by more than 50%. After withdrawal of long term androgen incubation AR increased again (75% of the control level). Cell population doubling time after long term incubation with androgen was considerably longer than of wild type cells, suggesting the selection of androgen receptor-poor cells during prolonged incubation with androgen.

AR mRNA was demostrated by Northern Blot hybridization. The reduction of the mRNA level was observed after treating the cells with 10 nM DHT for 6h and was more pronounced after prolonged incubation.

The data show down-regulation of the androgen receptor by its physiological ligand DHT.

Z 19

Characterization of Insulin-like growth factor I and II receptors in human lung cancer cell lines

M. Rotsch, C. Hennig, C. Erbil, M. Maasberg, K. Havemann, Dept. Int. Medicine, Div. Hematology/Oncology, Baldingerstrasse, D-3550 Marburg, Germany

The Insulin-like growth factor I and II have been identified as mitogenic for a variety of cell types including lung tumor cell lines. Small cell lung cancer (SCLC) and Non-Small cell lung cancer (NSCLC) cell lines were investigated for the IGF I and the IGF II receptor expression. The IGF I and the IGF II receptor were characterized by radioreceptor assays, chemical cross-linking and northern blot hybridization. All tested SCLC and NSCLC cell lines showed specific ^{125}I-IGF I binding with a high affinity (0.6-3 nM). ^{125}I-IGF II binds simultaneously to the IGF I and the IGF II receptors. The Scatchard analysis was linear although the binding had occurred at both receptors. Radioreceptorassays performed on membrane preparations of the cells gave evidence that ^{125}I-IGF II binds to a high (30-80 pM) and low (1-4nM) affinity site, which are differently distributed in subcellular fractions. The high affinity binding site was identified as IGF I receptor, the low affinity sites as IGF II receptor. Northern blot hybridization indicated that IGF I and IGF II mRNA was expressed by all tested cell lines. The IGF I binding was inhibited by an IGF I receptor antibody (α-IR3). The IGF II binding to its receptor was likewise inhibited by an IGF II receptor antiserum. The binding capacity and the affinity of ^{125}I-IGF II for the IGF II receptor was enhanced in the presence of mannose-6-phosphate.

Z 20

MALIGNANT TRANSFORMATION OF SECONDARY RAT EMBRYO FIBROBLASTS BY T24HA-RAS IS ASSOCIATED WITH AN ALTERATION IN OXYGEN CONSUMPTION

L.A. Kunz, A. Simm*, G. Adam*, and W. Mueller-Klieser; Inst. of Physiol. and Pathophysiol., Univ. of Mainz; *Faculty of Biology, Univ. of Konstanz.

The effect of T24Ha-ras transfection on cellular growth, volume and oxygen consumption (QO_2) was investigated using monolayer cultures of nontumorigenic, spontaneously immortalized (Rat1) and c-myc transfected (M1) rat embryo fibroblasts and an additionally T24Ha-ras transfected, tumorigenic clone of each (Rat1-T1 and MR1, respectively). Cellular growth and volume were quantified by standard methods. The respiratory activity of the cells was assessed by a photometric technique. T24Ha-ras-transfection of both Rat1 and M1 cells caused morphological alterations such as a statistically significant 30 - 50 % decrease ($p < 0.001$) in cell volume of both exponentially growing and plateau phase cells. Among other factors this effect might be responsible for the 8 and 5 times higher cell density of Rat1-T1 and MR1 cells in the plateau phase. Furthermore, an expected stimulation of the cellular growth after T24Ha-ras transfection was documented. In contrast, oxygen consumption rates of Rat1-T1 and MR1 were significantly lower ($p < 0,02$) than those of Rat1 and M1 cells with different kinetics during monolayer growth. Whereas the QO_2 of the tumorigenic cells decreased or stagnated throughout the entire period of plateau phase, a decrease in oxygen uptake was observed in Rat1 and M1 cells until day 4 - 5 only, followed by an increase with values almost returning to those of the early exponential phase. In conclusion, the transfection of Rat1 and M1 cells with T24Ha-ras oncogene alters not only cellular morphology and growth but also the oxygen metabolism. Supported by the DFG (Mu576/2-4)

Z 21

RAT MAMMARY ADENOCARCINOMA CELL CLONES OF DIFFERENT METASTATIC POTENTIAL REACT DIFFERENTLY TO TRANSFORMING GROWTH FACTOR BETA 1 (TGF-β1)

Andreas M. Kaufmann, Michael Stoeck, Volker Schirrmacher and Rosemarie B. Lichtner; Institut für Immunologie und Genetik, DKFZ, 69-Heidelberg.

The rat mammary adenocarcinoma system 13762NF consists of several clones of common origin but with differing metastatic potentials. The TGF-β's are involved as autocrine or paracrine factors in the control of growth and differentiation of many cells. In particular the production of extracellular matrix (ECM), proteases and their inhibitors are regulated by this factor, suggesting that TGF-β might play a role in metastasis. We therefore investigated the effects of porcine TGF-β1 on cell clones with defined metastatic potentials. TGF-β1 inhibited growth of nonmetastatic clone MTC ($IC_{50} \sim$ 30 pg/ml) but not of clone MTLn2 of intermediate and clone MTLn3 of high metastatic potential.

Low concentrations of TGF-β1 (1pg/ml) increased 3H-thymidine incorporation in MTLn3 cells. Upon TGF-β treatment all three clones exhibited morphological changes such as flattening of cells and reorganisation of actin fibers indicating altered adhesive properties. In MTLn2 cells TGF-β1 induced a reduction of cytokeratin content (no. 8, 14, 19). The same effects were achieved when untreated MTLn2 cells were plated on ECM produced by MTLn3 or TGF-β1 stimulated MTLn2 cells. These changes were reversible upon replating of cells. Our data suggest that metastatic tumor cells may modulate biological properties of adjacent cells by virtue of their ECM composition and/or local diffusible factors.

Z 22

Altered growth behaviour of human keratinocytes after ras-transfection

Hülsen A., Boukamp P., Game S.*, Donnelly M.*, Fusenig N. E. Abt. Carcinogenese und Differenzierung in vitro, DKFZ, 6900 Heidelberg, FRG; * Dept. of Oral Medicine, Dental Hospital, Bristol BS1 2LY, UK

Cells of a spontaneously immortalized human keratinocyte cell line (HaCaT) were transfected with the cellular Harvey ras-oncogene.
Only clones, which express the mutated ras-protein p21, were tumorigenic in nude mice, forming either benign tumors or malignant, invasive carcinomas. These tumorigenic clones had a higher plating-efficiency, viability and growth-rate than nontumorigenic clones under serum- and growth factor-free conditions in vitro. Serum withdrawal in addition to low cell densities caused a strong decrease of colony-forming-efficiency (<5%) of nontumorigenic cells, whereas tumorigenic cells, seeded at clonal densities, still formed colonies on an average efficiency of 16%. This increased growth autonomy of the malignant HaCaT-ras-clones might be due to higher expression of EGF-like factors (possibly TGF-α), as detected in confluent cultures under serumfree conditiones.
The classical soft-agar-test did not show any correlation to the transformed phenotype of these cells. On the contrary, while the parent HaCaT-line formed colonies in this semisolid medium, EJ-ras-transfected clones, even the malignant ones, had generally lost the ability of anchorage-independent growth.
So far, only one in vitro growth parameter correlated with in vivo malignancy: while TGF-β reduced growth-rates of nontumorigenic and benign HaCaT-clones in serumfree medium, the carcinoma-forming HaCaT-clones were either refractory or even stimulated by TGF-β.
Investigations on representative clones of the nontumorigenic, benign and malignant type showed that their TGF-β synthesis was not correlated to their TGF-β response, but that the refratory or stimulated clones expressed less TGF-β receptors than the inhibition-sensitive clones.

Z 23

Epidermal differentiation of Ha-ras transfected human HaCaT cells.

D. Breitkreutz, P. Boukamp, C. Ryle*, H.-J. Stark, N.E. Fusenig, Institut für Biochemie, DKFZ, Heidelberg; *Neuroscience Group, Molecular Medicine, University of Oxford, UK

Tumorigenic clones evolving from the human epidermal cell line HaCaT after transfection with c-Ha-ras oncogene (val-12) behaved differently when injected s.c. in nude mice. Malignant clones developed carcinomas and grew invasively in surface transplants [Boukamp et al., Cancer Res 50:2840-2847, 1990]. In culture, epidermal markers, such as keratins K1 and 10, were expressed at high cell density or upon depletion of retinoic acid (RA). Accordingly, all clones formed stratified epithelia in transplants with significant amounts of K1 and 10. Simple epithelial type keratins and K13 (common in internal stratified epithelia) were largely suppressed in vivo like upon RA depletion. In transplants of HaCaT cells all layers were stained by K1-antibodies (immunofluorescence), in contrast to the regular suprabasal location of K1 in normal transplants, while K10 appeared regularly in mainly suprabasal position. Tumorigenic clones showed, in addition to K1-/10-dissociation, a general shift of these keratins towards upper layers. Highly invasive cell masses reacted similarly, also comparable to corresponding carcinomas. Thus, differentiation potential was not inversely related to malignant properties, correlating to previous data on a series of mouse keratinocytes. [Breitkreutz et al., Eur J Cell Biol 42:255-267, 1986]

Z 24

Platelet Derived Growth Factor (PDGF) as Autocrine Growth Factor for a Human Melanoma Cell Line

Behl C., Winkler J., Apfel R., *Schlingensiepen K.-H.,* Brysch W., Bogdahn U.
Dept. of Neurology, Würzburg Univ., D-8700 Würzburg, F.R.G.
* MPI for Biophysics, D-3400 Göttingen, F.R.G.

The human melanoma cell line HTZ-19 was screened for the expression of different growth factors by northern hybridizations. The secretion of factors was tested by western blotting of the conditioned culture supernatants. A putative amplification of some growth factor genes was investigated by southern blotting.
HTZ-19 was positive for mRNAs of PDGF-A and -B-chains, TGF-β_1, IGF-I and bFGF and negativ for TGF-α, TGF-β_2, TGF-β_3, insulin and IGF-II. PDGF-AA-, TGF-β- and bFGF-peptides could be detected. Southern Blots showed no amplification of the PDGF-A-chain-, -B-chain- and bFGF-gene.
Although HTZ-19-cells growing under serum-free conditions only relatively minor amounts of PDGF-A-mRNA could be detected, expression of the PDGF-A-chain-gene was remarkably enhanced after incubation with purified PDGF. The highest expression level was seen after a 4h-PDGF-stimulation. This indicates a PDGF-induced autocrine stimulation involving PDGF-AA which could amplify the response to PDGF. After addition of purified PDGF to the cells their growth was stimulated as shown by ^{3}H-Thymidin incorporation (20% growth stimulation at 10ng/ml f.c. PDGF).
A 48h incubation of HTZ-19 with phosphorothioate-oligonucleotides as specific antisense against PDGF-A-chain-mRNA leads to a growth inhibition (approx. 60% at 0.25µM f.c. antisense).
These data suggest that PDGF may play an important role in growth stimulation of HTZ-19 melanoma cells and that PDGF may be a part of an autocrine growth regulatory loop.

Z 25

GENETIC, DEVELOPMENTAL AND MOLECULAR ANALYSIS OF THE TUMORSUPPRESSOR GENE LETHAL(2)TUMOROUS IMAGINAL DISCS l(2)tid OF DROSOPHILA MELANOGASTER

Kurzik-Dumke, U., Phannavong, B., Gundacker, D., Gateff, E.

Institute of Genetics, Johannes Gutenberg-University, 6500 Mainz, F. R. G.

A great number of lethal mutations in Drosophila interfere with imaginal disc development. Three tumorsuppressor Genes are instrumental in imaginal disc differentiation (Gateff,E. Science 200, 1448, 1978). One of these three genes called lethal(2) tumorous imaginal discs l(2)tid) was analysed (i) genetically, (ii) cytogenetically, (iii) anatomically, (iv) histologically and (v) developmentally. The gene is located on the giant chromosome II in the band 59F4-6. The homozygous mutant animals exhibit tumorous imaginal discs and die at the end of larval life.The differentiation capacity of the imaginal discs is limited to only cuticle and few hairs. Transplanted into the abdomen of wild-type flies the mutant pieces grow in a similar lethal, tumorous fashion as in situ. In order to determine the time of l(2)tid gene activity, we made double mutants between l(2)tid and three other imaginal disc mutants such as lethal(2)giant larvae (l(2)gl), lethal(2)fat^{gd} (l(2)ft^{gd}) and lethal(2) 37Cf (l(2)37Cf). The analysis of the morphology, histology and developmental capacities of the imaginal discs of these double mutants allowed us to determine the following order of gene expression:

l(2)37Cf l(2)gl l(2937Cf l(2)tid l(2)ft^{gd}

maternal zygotic expression

Z 26

DO METASTATIC VARIANT CELLS EXHIBIT DIFFERENCES IN THEIR CELL SURFACE LECTIN EXPRESSION?

S. Gabius[1], V. Schirrmacher[2], S.S. Joshi[3], H. Franz[1] and H.-J. Gabius[4]

[1]Medizinische Universitätsklinik Göttingen, [2]Deutsches Krebsforschungszentrum, Heidelberg, [3]University of Nebraska Medical Center, Omaha and [4]Max-Planck-Institut für experimentelle Medizin, Göttingen.

The expression of cell surface receptors is crucial to the biological behavior of tumor cells. Among diverse types of molecular recognition protein-carbohydrate interaction can be involved in mediation of cellular contacts. To quantify cell surface expression of sugar receptors (lectins) of native cells, chemical glycosylation of E. coli β-galactosidase is employed to generate a versatile glycocytological tool for this purpose (Gabius et al. (1989) Anal. Biochem. 182, 447-451). Model systems of strongly versus weakly metastatic cells including the lymphoma Eb/ESb, the lymphosarcoma RAW117 P/H10 and the melanoma B16 F_1/F_{10} were quantitatively assessed for cell surface binding using a panel of neoglycoenzymes, carrying components of the carbohydrate part of cellular glycoconjugates. Significant differences in receptor quantity were revealed, e.g. for galNAc-exposing probes. Differential binding of tumor cells to plastic-immobilized neoglycoproteins substantiated that such alterations can translate into notable changes of lectin-mediated adhesion. Moreover, modulation of lectin expression was seen after chemically induced differentiation. The measured differences may have a bearing on adhesive contacts during the metastatic process.

Z 27

ATTACHMENT OF HUMAN CHORIOCARCINOMA CELL SPHEROIDS TO UTERINE EPITHELIAL MONOLAYERS: QUANTITATIVE MEASUREMENTS OF ADHESION

N.J. John, R. Grümmer, and H.W. Denker
Inst. für Anatomie der Universität Essen

Understanding of cell-to-cell interactions responsible for the invasive property of cancer cells is still rudimentary. In particular, the parameters involved in intercellular adhesion need to be defined. As a model for the study of adhesion and invasion, human choriocarcinoma cell lines (BeWo and JAr) grown as multicellular spheroids and human epithelial endometrial cell lines (RL95-2, HEC-1-A, KLE, AN3-CA) grown as monolayers were used in an in vitro model system. As determined by MAIA or RIA, BeWo spheroids released the placental hormones human chorionic gonadotropin (hCG), progesterone (P), and 17-ß-estradiol (E_2) into the culture medium. JAr spheroids produced hCG and P. Indirect immunofluorescence of the endometrial cell lines revealed distinct patterns of structural determinants characteristic of epithelial cells (RL95-2: + α-keratin; HEC-1-A: + α-keratin and uvomorulin; KLE: + α-keratin and vimentin; and AN3-CA: + vimentin). Attachment of BeWo or JAr spheroids to monolayer cultures of the endometrial cell lines was quantified using a centrifugal force-based adhesion assay. Results showed similar patterns of attachment for both BeWo and JAr spheroids to the different endometrial cell monolayers (a) after 1 hr incubation: adhesion (measured following exposure to 12xg RCF) was greatest with cell line RL95-2 (>70% of the spheroids were attached compared to 50% for poly-d-lysine control), minimal with HEC-1-A (<10% attached), and null with KLE and AN3-CA and (b) after 24 hrs incubation: attachment increased over time (with the exception of JAr x AN3-CA where 0 attachment was maintained). Significant differences between BeWo and JAr were most notably measured when comparing adhesion (a) with HEC-1-A at 5 and 24 hrs (JAr > BeWo); and (b) with AN3-CA at 24 hrs (BeWo > JAr). This assay system has identified specific endometrial cell lines which are adhesive vs. non-adhesive for choriocarcinoma cells. Further experimentation to identify and study regulation of the specific intercellular adhesive molecules involved is now possible.

Z 28

OXYGENATION STATUS OF TUMOR SPHEROIDS ASSESSED BY SIMULTANEOUS PO_2 MICROELECTRODE MEASUREMENTS AND MISONIDAZOLE LABELING

M. W. Gross, U. Karbach and W. Mueller-Klieser
Inst. für Physiologie & Pathophysiologie der Universität Mainz

To quantify the oxygen dependence of MISO binding, oxygen tension (PO_2) profiles were recorded in EMT6/Ro tumor spheroids (mean diameter ± SD, 830 ± 30 µm) using O_2-sensitive microelectrodes immediately before and after a 1 hr incubation with 50 µmol/l ^{3}H-MISO. The distribution pattern of the misonidazoleadducts was then assessed by conventional autoradiography. The data showed a statistically significant (Mann-Whitney-U-test, $P < 0.005$) accumulation in spheroid regions with PO_2 values < 36 mmHg compared to the peripheral well oxygenated regions. Owing to an alteration of cellular respiration, the central oxygen tension values were elevated by 16 ± 8 mmHg (mean ± SD) during incubation with ^{3}H-MISO. When corresponding experiments were performed with spheroids (diameter 870 ± 16 µm, mean ± SD) in lowered oxygen tensions (40 mmHg) and increased glucose (4.5 g/l), the average central PO_2 was elevated by 4 ± 2 mmHg (mean ± SD). A significant increase in labeling in the inner parts compared to the peripheral spheroid regions could be observed at O_2 tensions below 8 mmHg ($P < 0.005$). The data suggest that MISO labeling may yield qualitative information about the presence of areas with relatively low oxygen tensions in malignant tissue but may not exclusively detect radiobiologically hypoxic cells with PO_2 values < 2 mmHg.
The results presented here are part of the doctoral thesis of M. W. Gross.
Supported by the DFG: Mu 576/2-4