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CIRRHOSIS AND CARCINOMA

Treatment of cirrhotic rats with epidermal growth factor and insulin accelerates liver DNA synthesis after partial hepatectomy

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Abstract Prevention of postoperative hepatic failure is important after hepatic resection. In patients with cirrhosis, impaired liver function and regenerative capacity after major hepatic resection are associated with increased morbidity and mortality. In this study, a combination of epidermal growth factor (EGF) and insulin were used as hepatotrophic factors in an attempt to stimulate DNA synthesis after 70% hepatectomy (HTX). Regenerative capacity was evaluated in normal and cirrhotic rat liver by measuring DNA synthesis *in vivo*. Micronodular liver cirrhosis was established by the simultaneous oral administration of CCl_4 and phenobarbital. Epidermal growth factor plus insulin was injected subcutaneously immediately after and 12 h after HTX or sham operation was performed. Rats were killed 24 h after the operation and liver regeneration was estimated by [^{3}H]-thymidine incorporation into DNA as well as an autoradiographic nuclear labelling index. Hepatectomy increased [^{3}H]-thymidine incorporation significantly in both normal and cirrhotic rats. In cirrhotic rats, [^{3}H]-thymidine incorporation after HTX was significantly lower than in normal rats and administration of a combination of EGF and insulin after HTX enhanced [^{3}H]-thymidine incorporation. In conclusion, DNA synthesis 24 h after HTX is decreased in cirrhotic rats compared with normal rats and EGF supplementation with insulin accelerates DNA synthesis in hepatectomized cirrhotic rats. The data suggest that administration of combinations of exogenous hepatotrophic factors may play a useful role in the treatment of cirrhotic patients undergoing major hepatic resection.

Key words: epidermal growth factor, experimental liver cirrhosis, insulin, liver regeneration.

INTRODUCTION

Partial hepatic resection is a mainstay of treatment for liver tumours; however, many tumours arise in the setting of cirrhosis and diminished hepatic reserve. Normal liver usually has sufficient regenerative capacity to permit resection of up to 70% of the total liver cell mass. In liver cirrhosis, impaired liver function and poor regenerative capacity after hepatectomy (HTX) leads to increased morbidity and mortality.[1,2] There have been few attempts to improve hepatic regeneration in the cirrhotic liver.[3,4] Utilizing a rat liver model of micronodular cirrhosis, established by the simultaneous oral administration of carbon tetrachloride (CCl_4) and phenobarbital, supplemental transforming growth factor alpha (TGFα) enhanced liver regeneration as measured by *in vivo* [^{3}H]-thymidine incorporation into DNA.[4] Epidermal growth factor (EGF) is an hepatotrophic factor which promotes DNA synthesis in rat isolated hepatocytes and regenerating rat liver after 70% HTX[5–7] and which shares amino acid homology with TGFα.[8] Epidermal growth factor has been shown to have synergistic effects on liver regeneration when supplemented with insulin or glucagon in normal rats *in vivo*.[7] To determine if EGF alone or in combination with insulin increased DNA synthesis in cirrhotic rat liver, hepatic regeneration was evaluated by measurement of liver DNA synthesis using two independent parameters, [^{3}H]-thymidine incorporation into DNA and a nuclear labelling index.[4,9]

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METHODS

Chemicals

Epidermal growth factor (fragment 20–31; EGF) and bovine insulin were purchased from Sigma Chemical Co. (St Louis, MO, USA). [Methyl-^{3}H]-thymidine (37 MBq/mL) was from Amersham (Arlington Heights, IL, USA).

Establishment of cirrhosis in a rat model

Male Wistar rats (150–200 g) were obtained from Charles River Laboratories (Wilmington, MA, USA). Micronodular cirrhosis was established according to the method of Proctor and Chatamra, by the simultaneous oral administration of CCl_4 and phenobarbital, which increases the sensitivity of the liver to CCl_4 by induction of the microsomal hydroxylating enzyme system.[10] Rats were given phenobarbital sodium (35 mg/dL) in drinking water. After treatment for 10–14 days with phenobarbital sodium, the rats weighed 250–300 g. The rats were anaesthetized with isoflurane and oxygen anaesthesia[11] and CCl_4 was administered once a week at midday using a curved feeding needle (16 gauge). After the beginning of CCl_4 feeding, bodyweight was measured daily. The initial dose of CCl_4 was 0.04 mL and successive doses were administered according to daily weight fluctuations in each rat. If bodyweight increased, the dose of CCl_4 was increased 0.04 mL per week. If bodyweight decreased, administration of CCl_4 was decreased to 0.02 mL or stopped for the week. Administration of CCl_4 lasted for 10 weeks and phenobarbital was continued throughout the period of CCl_4 gavage. Both CCl_4 and phenobarbital were discontinued at least 2 weeks before the operation.

Partial hepatectomy and sham operation

Cirrhotic and normal rats were anaesthetized by isoflurane/O_2 anaesthesia[11] and subjected to sham operation or 70% hepatectomy. Sham rats were subjected to laparotomy and manipulation of the liver. Hepatectomy was performed after the method of Higgins and Anderson between 08:00 and 12:00 h and resected liver was weighed.[12]

Measurement of DNA synthesis

Sham-operated and hepatectomized normal and cirrhotic rats were randomly placed in one of four groups. Group A, sham operated, no treatment (normal rats $n=9$, cirrhotic rats $n=6$); group B 70% HTX, no treatment (normal rats $n=9$, cirrhotic rats $n=7$); group C 70% HTX, subcutaneous injection of EGF (30 nmol/kg) just after and 12 h after 70% HTX (normal rats $n=3$, cirrhotic rats $n=3$); group D 70% HTX, subcutaneous injection of EGF and insulin (30 nmol/kg and 0.2 nmol/kg) just after and 12 h after HTX (normal rats $n=4$, cirrhotic rats $n=5$).

All rats were killed 24 h after the operation and the remnant liver and spleen were excised and weighed. Blood samples were collected from the inferior vena cava and serum albumin, total bilirubin and glucose were measured using commercial kits (Sigma Chemical Co., St Louis, MO, USA). Serum insulin was measured by radioimmunoassay as performed by the Michigan Diabetes Research and Training Center. Liver DNA synthesis 24 h after hepatectomy was assessed using [^{3}H]-thymidine incorporation. [^{3}H]-Thymidine was injected intravenously 1 h before the rats were killed.[4,8] A 0.5 mL aliquot of liver homogenate (using a Dounce-type homogenizer; Sigma) was precipitated with ice-cold 10% trichloroacetic acid twice and 70% ethanol once. [^{3}H]-Thymidine radioactivity in the precipitate was measured with a Beckman LS 6000LL liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA, USA). A separate 0.1 mL aliquot was analysed to measure DNA using a modified diphenylamine reaction for desoxypentose.[13] Data were expressed as c.p.m./μg DNA. To minimize variations in the specific activity of the lots of [^{3}H]-thymidine used, [^{3}H]-thymidine incorporation into DNA was expressed as a percentage of [^{3}H]-thymidine incorporation into DNA of sham-operated, non-cirrhotic rat liver.

To corroborate the observed results of [^{3}H]-thymidine incorporation into DNA, autoradiographic analysis was performed and a nuclear labelling index was calculated. Tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin blocks. Sections were cut, dipped in Kodak NTB-3 emulsion (Eastman Kodak, Rochester, NY, USA) and exposed for 4 weeks. Slides were developed in Dektol, counterstained with haematoxylin and eosin and analysed under the microscope with a 40× objective lens. Approximately 1000 nuclei were scored per experimental condition and the percentage of total nuclei positive for silver grains was used to calculate the labelling index.

Statistical analysis

All values shown represent the mean ± SEM. Student's t-test was used for comparing group means. The level of significance was accepted at $P<0.05$.

RESULTS

Establishment of liver cirrhosis in rats

Thirty-four rats were treated with CCl_4 and phenobarbital. These rats gained significantly less weight than untreated rats (Fig. 1). Overall mortality was 18% (6/34). All animals treated had macroscopic changes of the liver compatible with micronodular cirrhosis. Ascites developed during CCl_4 treatment in five treated rats (15%) but disappeared when the dose of CCl_4 was reduced or stopped. Three treated rats (9%) were noted to have ascites at the time of operation. Abdominal wall or intra-abdominal collateral vessels or a dilated splenic

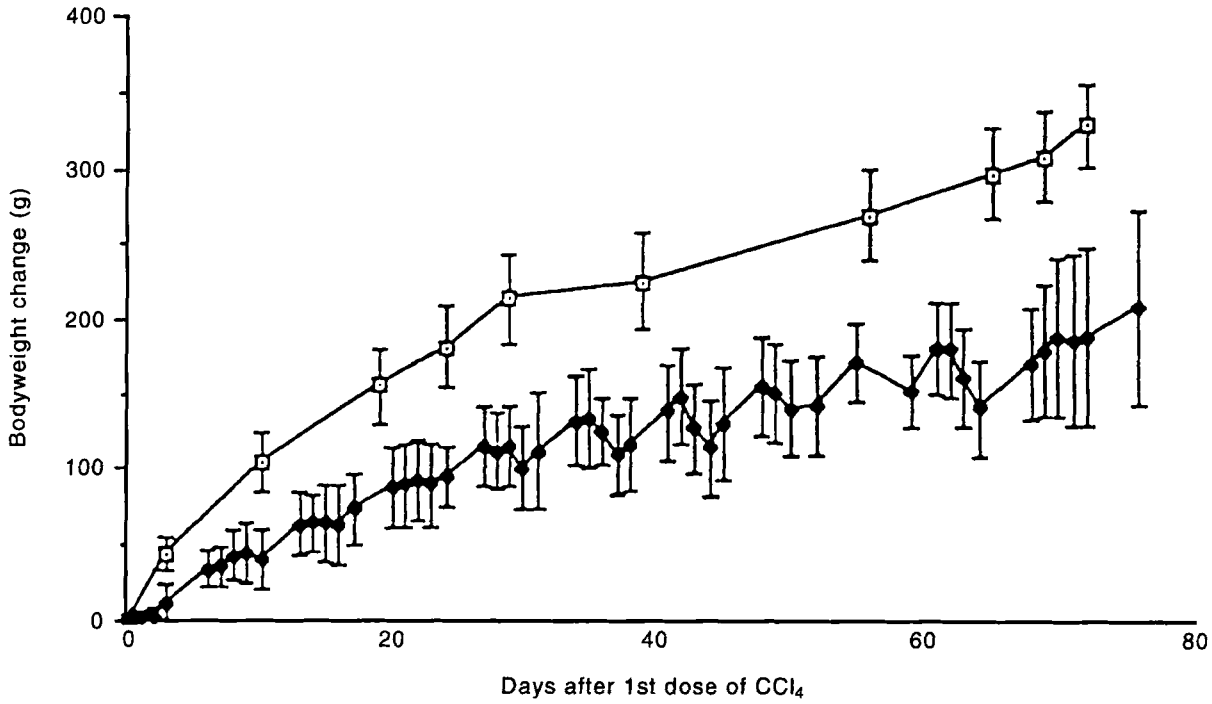

Figure 1 Bodyweight change in experimental animals. All rats were weighed daily after beginning the administration of carbon tetrachloride (CCl_4). Bodyweight change, compared with starting bodyweight, is expressed on the ordinate. Points are means of cirrhotic rats (◆, $n=16$) or normal controls (□, $n=5$). Error bars represent SEM.

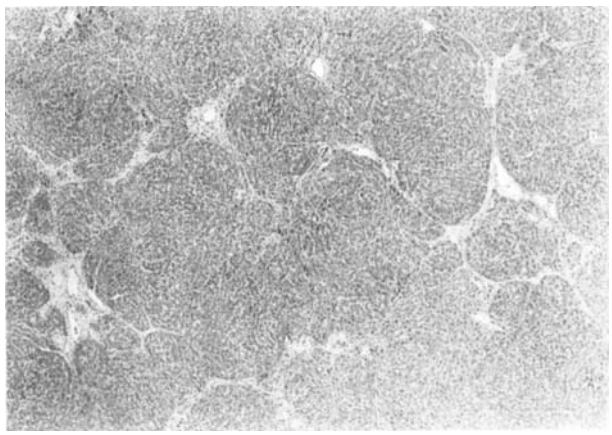

Figure 2 Histology of CCl_4-treated cirrhotic rat liver. This section of liver demonstrates established micronodules of liver separated by the thick bands of fibrous scar (Mallory's trichrome stain).

vein were observed in all rats. The overall effectiveness of this method for creating liver cirrhosis was 82% (28/34). Figure 2 is representative of the typical microscopic features of cirrhosis found in these rat livers.

Characteristics of rats with liver cirrhosis

To corroborate the gross and microscopic features of cirrhosis in the treated rats, selected biological and biochemical parameters were measured (Table 1). The splenic weight of cirrhotic rats was greater than normal rats. Serum albumin of cirrhotic rats at time of death was less than normal rats. The serum total bilirubin of cirrhotic rats at death was greater than in normal rats.

Table 1 Biological and biochemical parameters of experimental animals

	Normal rats ($n=25$)	Cirrhotic rats ($n=21$)
Bodyweight (g)	589±12	463±19*
Anterior lobe of liver (g/100 g bodyweight)	2.51±0.10	2.54±0.16
Spleen (mg/100 g bodyweight)	220±8	286±28*
Albumin (g/dL)	4.00±0.20	3.41±0.16*
Total bilirubin (mg/dL)	0.15±0.05	0.40±0.10*

Data are expressed as mean±SEM. *$P<0.05$ versus normal rats.

Insulin levels in normal or cirrhotic rats subjected to 70% hepatectomy treated with EGF alone or EGF plus insulin were lower than in normal or cirrhotic sham-operated rats (Table 2). Subcutaneous injection of insulin (0.2 nmol/kg) had no effect on plasma insulin levels between EGF-treated rats and EGF plus insulin-treated rats. The serum glucose level after death is shown in Table 2. The glucose level of rats treated with 70% HTX, EGF and insulin was significantly less than in normal sham rats.

Table 2 Serum insulin and glucose level 24 h after operation

	Insulin (μunits/mL)			Glucose (mg/dL)		
	Sham	HTX+EGF	HTX+EGF+Ins	Sham	HTX+EGF	HTX+EGF+Ins
Normal rats	12.6±1.7	6.2±0.6*	5.0±1.0*	156±9	136±3	113±14*
Cirrhotic rats	18.8±2.7	7.6±0.9*	8.8±1.0*	143±1	134±13	123±14

Data are expressed as mean±SEM. *$P<0.05$ versus sham-operated rats in each group. HTX, 70% hepatectomy; EGF, epidermal growth factor; Ins, insulin.

DNA synthesis after sham operation and 70% hepatectomy

Twenty-four hours after 70% HTX, [^{3}H]-thymidine incorporation into the liver of normal rats was significantly increased from 100±3% (normal sham) to 928±148%, which was 9.3-fold that of normal sham rats (Fig. 3a). In rats with normal liver, exogenous EGF without supplemental insulin did not enhance [^{3}H]-thymidine incorporation after 70% HTX (857±101%), whereas exogenous EGF with insulin did (1625±519%), but not significantly (Fig. 3a). After 70% HTX in cirrhotic rats, [^{3}H]-thymidine incorporation into the liver also significantly increased from 77±8% (cirrhotic sham) to 284±59% (cirrhotic HTX), 3.7-fold that of cirrhotic sham rats (Fig. 3b). These levels of [^{3}H]-thymidine incorporation were significantly lower than in rats with normal liver (928±148%). The combination of exogenous EGF and insulin significantly improved [^{3}H]-thymidine incorporation in cirrhotic rat liver (284±59 *vs* 706±186%). Exogenous EGF alone had no growth-promoting effect on cirrhotic liver after 70% HTX (204±57% *vs* 284±59%).

Thymidine autoradiography

To support the results of [^{3}H]-thymidine incorporation, autoradiography was performed and a nuclear labelling index was calculated for both normal and cirrhotic rat liver. Over 1000 nuclei were analysed in tissue sections of individual or duplicate rats with normal or cirrhotic liver subjected to sham or 70% HTX. In rats with normal liver subjected to sham or 70% HTX, the labelling index was < 1 and 23%, respectively. Qualitatively, no positive nuclei were seen in cirrhotic rats subjected to sham hepatectomy. In cirrhotic liver from 70% HTX, the labelling index was 5%. A further marked increase in the labelling index of liver from hepatectomized cirrhotic rats treated with supplemental EGF and insulin (14%) was also noted. Representative autoradiographs of regenerating cirrhotic rat liver treated with EGF and insulin or without treatment are shown in Fig. 4(a,b).

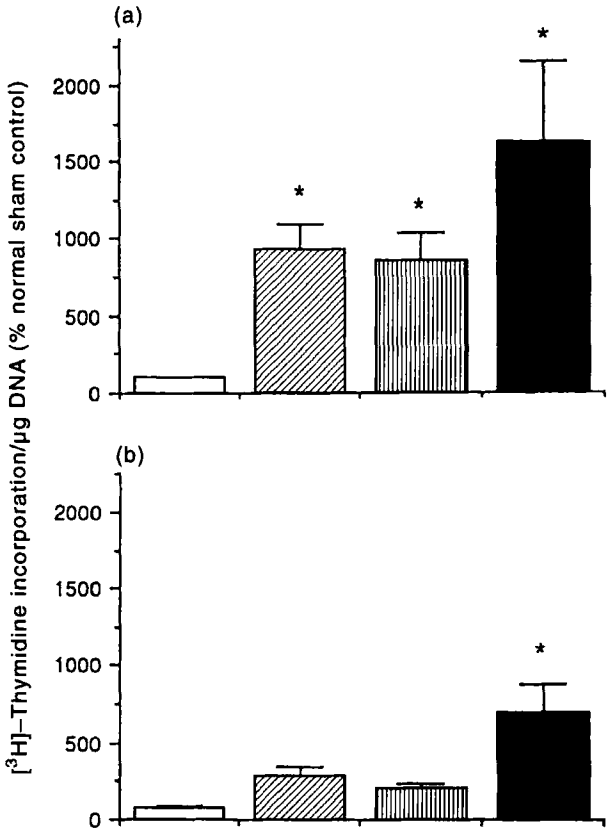

Figure 3 (a) [^{3}H]-Thymidine incorporation into normal rat liver 24 h after hepatectomy. In rats with normal liver, [^{3}H]-thymidine incorporation was significantly increased when sham hepatectomy (□) was compared to 70% hepatectomy (HTX, ▨; *$P<0.05$). Exogenous EGF with (■) or without (▥) insulin was not significantly different from controls. (b) [^{3}H]-Thymidine incorporation into cirrhotic rat liver 24 h after hepatectomy. In hepatectomized cirrhotic rats, [^{3}H]-thymidine incorporation into saline-treated liver was significantly increased from 77±8% (sham, □) to 284±59% (70% HTX, ▨; $P<0.05$. Exogenous EGF plus insulin (■) increased [^{3}H]-thymidine incorporation to 706±186% when compared with saline-treated cirrhotic liver subjected to 70% HTX (*$P<0.05$). Exogenous EGF alone (▥) had no significant effect on [^{3}H]-thymidine incorporation into cirrhotic liver after 70% HTX.

DISCUSSION

Previous experiments using the cirrhotic rat model described in this report have shown a beneficial effect of transforming growth factor α (TGFα) on DNA synthesis after 70% HTX.[4] The phenobarbital/CCl_4 cirrhotic rat model used in the present study was relatively easy to create and established micronodular cirrhosis with a high yield.[10] The characteristic features of liver histology, splenomegaly and dilated portal collateral cir-

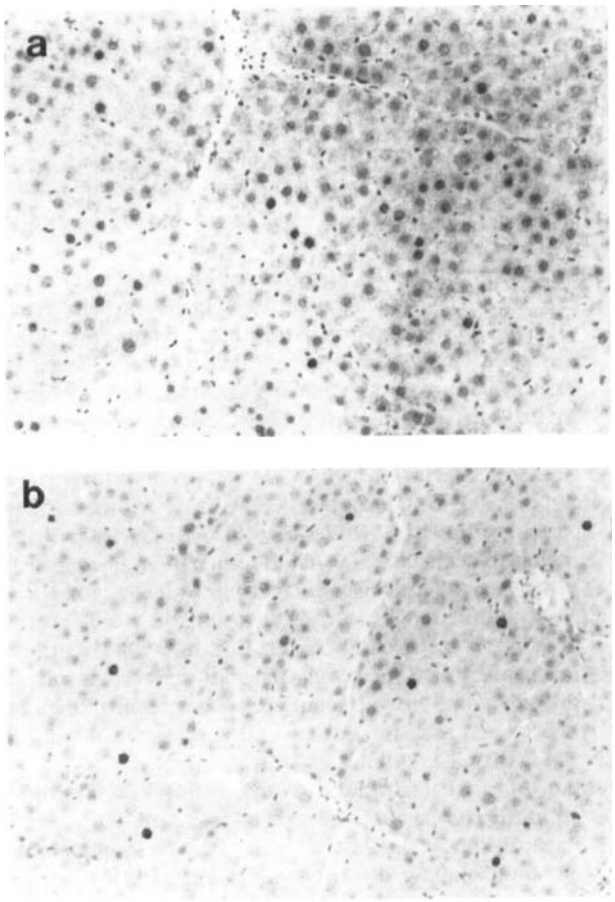

Figure 4 Autoradiographs of [^{3}H]-thymidine incorporated into cirrhotic rat liver. Representative autoradiographs of 70% hepatectomized cirrhotic rat liver treated with (a) epidermal growth factor and insulin or (b) without treatment.

culation were similar to those seen in humans with cirrhotic liver and portal hypertension. Hypoalbuminaemia and hyperbilirubinaemia are compatible with impaired liver function as well as structure. In addition, the present study confirms impaired liver DNA synthesis after 70% HTX in this cirrhotic rat model.

In normal liver, the rate of DNA synthesis after 70% HTX reaches a maximum at 24 h after operation and in cirrhotic liver the process of liver regeneration after 70% HTX was prolonged.[1,2,14] In this study, [^{3}H]-thymidine incorporation into DNA 24 h after 70% HTX was used to evaluate liver regeneration. This parameter has been established to be a useful indicator of the effect of growth factors on cell proliferation in a variety of experimental systems.[3,4,9] To minimize variation in the specific activity of the lots of [^{3}H]-thymidine used, [^{3}H]-thymidine incorporation into DNA was expressed as a percentage of [^{3}H]-thymidine incorporation into DNA of sham-operated normal rat liver and liver regeneration in both normal and cirrhotic rats could be evaluated quantitatively.

We hypothesized that EGF could be used as an hepatotrophic factor in cirrhotic rat liver. In the experiments reported here, the combination of EGF and insulin enhanced liver DNA synthesis in cirrhotic rats. Somewhat unexpectedly, treatment of rats with EGF alone (30 nmol/kg) did not result in improved DNA synthesis after 70% HTX in normal or cirrhotic rats, which is consistent with the results of Olsen *et al.*[7] Epidermal growth factor required supplemental insulin or glucagon to accelerate liver DNA synthesis after hepatectomy in normal rats;[7] hence, treatment with EGF and insulin were used in this study. We chose the dose of EGF, 30 nmol/kg, in accordance with the literature.[7] Rasmussen *et al.* reported that a higher dose of EGF (48 nmol/kg) was ineffective, but that smaller doses can stimulate liver regeneration: the cause of this has not been elucidated.[15] Two normal non-hepatectomized rats (normal sham) were treated with EGF (30 nmol/kg) and insulin (0.2 nmol/kg) and had a [^{3}H]-thymidine uptake of 84 ± 17%, which indicated that this treatment did not stimulate DNA synthesis in non-HTX rats.

Although insulin is an hepatocyte growth modulator, a number of lines of evidence suggest that treatment with insulin alone has limited or no benefit in normal regenerating liver.[15] Insulin is considered to be essential in liver regeneration and is transported into liver during the process of liver regeneration.[16] Insulin levels are reported to be high in cirrhotic patients and peripheral glucose metabolism suggests an impaired insulin response.[17,18] Insulin-stimulated glycogen formation has been shown to be markedly impaired.[19] D'Arville *et al.* reported that insulin levels of CCl_4/phenobarbital-induced cirrhotic rats were not significantly altered when compared with phenobarbital-treated rats.[20] Shankar *et al.* reported that insulin levels in thioacetamide-induced cirrhotic rats were significantly higher than in normal rats.[21] There may be a disturbance of the uptake of insulin in cirrhotic liver, possibly by receptor-mediated mechanisms.[21] After partial hepatectomy, insulin levels have been reported to decline and the administration of exogenous insulin results in little or no regenerative capacity.[8,16] In normal rats, decreased insulin and elevated glucagon levels have characterized the hepatic proliferative state.[16] In the present study, insulin levels in hepatectomized rats were significantly lower than in sham-operated rats with normal or cirrhotic liver and serum insulin and glucose levels were measured in treated and untreated normal and cirrhotic rats and found to be unchanged by the doses of insulin administered. Confirming the findings of the previously mentioned studies, insulin levels in cirrhotic sham rats were increased when compared with normal sham rats. The hyperinsulinaemia seen in these cirrhotic rats was not observed to have a cooperative effect with EGF in the acceleration of DNA synthesis. With regards to the effect of insulin alone on liver regeneration, one cirrhotic rat, treated preliminarily only with insulin (0.2 nmol/kg), had a [^{3}H]-thymidine uptake level of 398% of the normal sham-control rat compared to saline-treated hepatectomized cirrhotic rats at a level of 284 ± 59%. This was in contrast to the data on EGF and insulin, which gave a thymidine uptake value of 706 ± 186%. Due to this preliminary result and hyperinsulinaemia in cirrhotic rats, we did not use insulin alone on cirrhotic rats.[7,18]

There are few studies which correlate the effect of hepatotrophic factors in cultured hepatocytes and *in*

vivo studies. In monolayer cultures of isolated hepatocytes, EGF alone has a stimulatory effect on DNA synthesis and EGF supplemented by insulin markedly accelerates DNA synthesis.[5,22] The observed differences in the effects of EGF on isolated hepatocytes and hepatocytes *in vivo* are not well understood. Cellular density, or contact inhibition, the hormonal milieu and innervation of the liver *in situ* and damage during cell isolation might all play a role in the divergent responses seen in isolated hepatocytes and liver *in vivo*.

Epidermal growth factor and TGFα are similar in that they share a 33–44% homology in amino acid sequence and bind the same cell surface receptor.[7,23] Transforming growth factor α has a limited effect on liver regeneration in normal rats, but does have a stimulatory effect in cirrhotic liver.[4] Epidermal growth factor alone has no effect in cirrhotic liver. *In vivo*, TGFα, acting in an autocrine manner, appears to be the physiological peptide hormone and EGF, acting via either an endocrine or paracrine mechanism, also appears to be the physiological peptide hormone.[24,25] The reasons for the disparity in the effects of EGF and TGFα are not known. The effects of EGF and TGFα on liver regeneration cannot be quantitatively compared, because the true physiological doses are not known. Further, although EGF and TGFα bind the same receptor in plasma membranes,[8] there is evidence that EGF and TGFα have different post-receptor pathways of signal transduction. Insulin appears necessary for normal signal transduction by EGF.[26,27] It is tempting to speculate that the transforming properties of TGFα *in vitro* may be related to the lack of a requirement for additional regulatory factors, such as insulin. One potential drawback in the use of these growth factors in clinical trials is the potential for neoplastic transformation. Undetectable neoplasm or metastases in the remnant liver might be stimulated to grow by treatment of growth factors.

In conclusion, DNA synthesis 24 h after 70% HTX was significantly decreased in cirrhotic rats compared with normal rats. Combined treatment with EGF and insulin accelerated DNA synthesis in hepatectomized cirrhotic rats. Ultimately, such a combination of growth factor therapy may prove useful in the support of cirrhotic liver where regeneration must occur to allow patient survival.

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