THE JOURNAL OF GENE MEDICINE
J Gene Med 2005; **7**: 288–296.
Published online 27 October 2004 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jgm.661

RESEARCH ARTICLE

Adenovirus binding to cultured synoviocytes triggers signaling through MAPK pathways and induces expression of cyclooxygenase-2

Leslie J. Crofford[1]*
Kevin T. McDonagh[2]
Sutang Guo[1]
Hemal Mehta[1]
Huimin Bian[1†]
Lilli M. Petruzelli[2]
Blake J. Roessler[1]

[1]*Division of Rheumatology, Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109, USA*

[2]*Division of Hematology and Oncology, Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109, USA*

*Correspondence to: Leslie J. Crofford, Room 5510E, MSRB I, 1150 W. Medical Center Drive, Ann Arbor, MI 48109-0680, USA.
E-mail: crofford@umich.edu

[†]Current address: University of Indiana, Indianapolis, IN 46202, USA.

Received: 16 April 2004
Revised: 6 June 2004
Accepted: 18 June 2004

Abstract

Background Recombinant adenovirus can be administered *in vivo* to achieve transduction of a number of cell types including human synoviocytes. Immunogenicity of adenoviruses has limited their utility as vectors for gene delivery; however, specific mechanisms underlying the acute inflammatory response to adenovirus are not well understood. Activation of a number of signal transduction pathways occurs rapidly upon adenovirus binding to cell-surface receptors. We investigated stimulated expression of mitogen-activated protein kinases (MAPKs), cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) in human primary synovial fibroblasts to adenovirus expressing the *E. coli* β-galactosidase gene.

Methods Cultured rheumatoid synoviocytes were exposed to transduction-competent Ad/RSVlacZ recombinant adenovirus or transduction-incompetent (psoralen/UV-irradiated) Ad/RSVlacZ. The effects on COX-2 expression, PGE_2 levels and MAPK signaling in synoviocytes were assessed using a combination of reverse-transcription polymerase chain reaction amplification and immunoblotting.

Results Adenovirus treatment of synoviocytes increased levels of COX-2 mRNA and protein as well as PGE_2. Psoralen-treated transcriptionally inactive adenovirus was equivalent to untreated adenovirus for early COX-2 induction suggesting that viral genes were not required. Adenovirus treatment stimulated phosphorylation of ERK-1/-2, p38 MAPK, and JNK. Inhibition of the ERK and p38 MAPK pathways inhibited COX-2 expression and PGE_2 production.

Conclusions Taken together, these data demonstrate that a MAPK-dependent increase in COX-2 results in local prostaglandin production. These findings have clinical implications for use of adenovirus as vectors for *in vivo* gene delivery. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords adenovirus; cyclooxygenase-2; synoviocytes; MAPK

Introduction

Recombinant adenoviruses can be administered *in vivo* to achieve transduction of a number of cell types including synoviocytes, chondrocytes and fibroblasts [1–5]. Recombinant adenoviruses have advantageous features for *in vivo* administration including the ability to infect non-dividing cells and high efficiencies of transgene expression [6–8]. It has been posited that

transduction of synovial cells *in vivo* with recombinant adenoviruses to induce overexpression of anti-inflammatory or immunomodulatory proteins may be an innovative approach to the treatment of rheumatic diseases. Several groups have reported pre-clinical animal studies using *in vivo* administration of recombinant adenoviruses that represent models of intra-articular gene therapy for rheumatic diseases [9–15].

Immunogenicity is a significant limitation to the *in vivo* administration of recombinant adenovirus. An acute host immune response occurs rapidly following parenteral administration of virus and, in the case of intravascular injection, the sequelae can be catastrophic [16]. The viral capsid and genome (via expression of viral proteins) are both important mediators of the immune response to adenovirus [8,17–27].

The specific mechanisms underlying the acute inflammatory response to adenovirus infection are not completely understood. Activation of a number of signal transduction pathways occurs rapidly upon adenovirus binding to cell-surface receptors. Adenovirus capsids interact with both the Coxsackie adenovirus receptor (CAR) and the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins via the fiber knob protein and the penton protein RGD motifs, respectively. Expression of CAR correlates with the ability of adenovirus to infect target cells in a number of cell systems [28–32]. CAR is composed of an extracellular domain containing two disulfide-linked loops, a hydrophobic transmembrane domain, and a cytoplasmic domain. Functional analysis demonstrated that despite conservation of the cytoplasmic domain, the extracellular domain is sufficient to permit virus attachment [29,33]. Since the cytoplasmic domain of CAR is not required for virus entry, it is likely that other cell-surface receptors are critical for signaling events stimulated by adenovirus infection. The α_v integrins have been shown to facilitate internalization into cells [34]. Adenovirus internalization and infection is inhibited by the penton base, soluble RGD peptides, and function-blocking monoclonal antibodies directed against the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins [34]. It was subsequently shown that these cell-surface integrins, particularly $\alpha_v\beta_5$, are necessary for efficient adenovirus-mediated gene transfer [35–39].

Viral entry and nuclear localization have been shown to require the preservation of key signaling pathways and the induction of cell changes that mimic those seen in response to growth factors and integrins [40]. Viral endocytosis is dependent on phosphatidylinositol-3-OH kinase (PI3-kinase) activity, the small GTPases RacI and Cdc42, phosphorylation of p130 Crk-associated substrate ($p130^{CAS}$), and alterations in the actin cytoskeleton [41–43]. Activation of intracellular signaling pathways is also critical for the nuclear targeting of adenovirus. Adenovirus activates two distinct pathways, protein kinase A and p38 mitogen-activated protein kinase (MAPK), to facilitate transport to the nucleus [44].

The MAPK signaling pathways also play important roles in regulating gene expression [45]. The MAPKs include extracellular signal related kinases (ERK)-1 and -2, c-Jun N-terminal kinase (JNK), and p38. ERKs are typically activated by mitogens such as growth factors and hormones, while JNK and p38 are stimulated by stress stimuli including pro-inflammatory cytokines. Receptor-associated GTP transfer proteins (GTPases) trigger a cascade of protein kinases beginning with MAP kinase kinase kinases (MAPKKK), which activate MAP kinase kinases (MAPKK) by serine and threonine phosphorylation. MAPKKs are dual-specificity kinases that phosphorylate MAPKs on threonine and tyrosine residues. Once activated, MAPKs can directly phosphorylate transcription factors or transcriptional co-regulators or phosphorylate downstream kinases that also regulate transcription and mRNA stability.

Among the genes activated via MAPK signaling pathways is the inducible form of cyclooxygenase (COX), COX-2, by stimulating transcription and increasing mRNA stability [46–52]. COX is the pivotal enzyme responsible for conversion of arachidonic acid to prostaglandins. There are two isoforms of COX, COX-1 and COX-2. COX-1 is constitutively expressed in most cell types while the basal expression of COX-2 is highly restricted. COX-2 is rapidly up-regulated by many different inflammatory, mitogenic, and stress stimuli [53]. In most inflammatory settings including arthritis, prostaglandin E_2 (PGE_2) is the most abundant product of the COX pathway [54]. PGE_2 has pleitropic activities mediated by binding to G-protein-linked cell-surface receptors [55]. Injection of PGE_2 recapitulates the cardinal signs of acute inflammation via vasodilation with plasma extravasation and sensitization of nociceptors [56]. In addition to the role of PGE_2 in mediating classical inflammation, prostaglandins are important modulators of the innate immune system [57].

The MAPK signaling pathways are thought to be involved in induced expression of some inflammatory mediators by adenovirus infection. For example, Bruder and Kovesdi reported that adenovirus infection stimulated activation of the Raf/MAPK pathway leading to rapid induction of IL-8 expression in HeLa cells [6]. The phosphorylation of MAPK and Raf occurred within 10–20 min and heat inactivation of the virus eliminated MAPK pathway activation.

We hypothesized that adenovirus infection of synoviocytes could directly induce expression of inflammatory mediators including COX-2-derived PGE_2. In order to evaluate this hypothesis, we studied induction of signal transduction pathways and inflammatory mediator production in primary human synoviocytes from patients with rheumatoid arthritis (RA) after exposure to adenovirus expressing the gene for *E. coli* β-galactosidase. We now demonstrate that adenovirus infection induces expression of COX-2 and increases PGE_2 production. Furthermore, increased expression of these mediators is a result of activation of MAPK signaling pathways.

Materials and methods

Synoviocyte culture

Synovial tissues were obtained from patients with RA at the time of joint replacement surgery as approved by the institutional review board. Tissues were dissociated by mincing and then incubating in 4 mg/ml type I collagenase in Dulbecco's modified Eagle's medium (DMEM) for 4 h at 37 °C in an atmosphere containing 5% CO_2 [58]. Dissociated cells were cultured in DMEM supplemented with 10% human AB serum (Biowhittaker, Walkersville, MD, USA), 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA), penicillin (100 units/ml), and streptomycin (100 μg/ml). Primary synoviocytes were used between the 3rd and 8th passages.

Adenoviral vector preparation

Replication-defective adenoviral vectors (type 5) were deleted of sequences spanning E1A, E1B (map units 1–9), and a portion of the E3 region (map units 83–85), impairing the ability of this virus to replicate or transform nonpermissive cells [5,59]. In the vector Ad/RSVLacZ, the long terminal repeat of the Rous sarcoma virus drives transcription of the *E. coli* β-galactosidase (LacZ) gene with an SV40 polyadenylation sequence cloned downstream from the reporter [5].

High titer recombinant adenovirus was prepared by amplification in the permissive 293 cell line using established methods [60]. Virus was purified from cell lysates twice by cesium chloride gradient ultracentrifugation followed by desalting on Sephadex G-50 columns with phosphate-buffered saline (PBS). Titers were determined by OD_{260}, and were $\sim 1 \times 10^{13}$ particles/ml $\pm$ 1 log (1.0 OD unit $= 1.0 \times 10^{12}$ particles/ml). The viral preparations were analyzed for their ability to form plaques on confluent 293 cell monolayers [61]. Approximately 1 in 100 virus particles were infection-competent.

Inactivation of Ad/RSVLacZ

Ultraviolet (UV) light treatment in the presence of 8-methoxypsoralen was used to inactivate Ad/RSVLacZ [22]. 8-Methoxypsoralen (8-MP, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in dimethyl sulfoxide at 33 mg/ml and added to the high titer viral suspension to yield a final concentration of 330 μg/ml. The adenovirus with 8-MP was placed into a transparent container (Slide-A-Lyzer 10K dialysis cassette, Pierce, Rockford, IL, USA), exposed to UVA band light generated by a fluorescent tube at a distance of 1 cm above the virus. The UVA exposure was performed at 4 °C for a period of 30 min, resulting in an UVA dose of greater than 150 J/cm^2. The container was rotated periodically to maintain adequate cooling and ensure even exposure of the particles. The virus was then dialyzed extensively against Tris-EDTA buffer, pH 8.0, to remove unincorporated psoralen. Confirmation of adenoviral genome inactivation was determined in 293 cells in a series of limiting dilution infections followed by X-gal staining 48 h following infection.

Synoviocyte infection

Synoviocytes (5×10^4/well) were plated in 6-well plates the day prior to infection. Complete media was removed and cells were washed with PBS. Ad/RSVLacZ was added in 400 μl RPMI at a concentration of 10^4 transfection-competent virus particles per cell. Addition of virus was T = 0 for timed samples. Complete media to a final volume of 2 ml was added at 2 h after infection for longer incubations. MAPK inhibitors were added 10 min prior to addition of virus.

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was prepared by using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA). cDNA was prepared by reverse transcription as previously described [62]. PCR reactions were performed in 50 μl containing 5 μl cDNA (diluted 1 : 10 after reverse transcription of 5 μg total RNA), 10 mM Tris HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 50 μM dNTPs, 0.5 μl [α^{32}P]dCTP (3,000 Ci/mmol, Amersham), and 0.025 μl *Taq* polymerase (Perkin-Elmer-Cetus, Norwalk, CT, USA). Oligonucleotide primers (100 ng) were included in the reaction with sequences as follows: COX-2 sense 5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′ and antisense 5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′; IL-8 sense 5′-AAA CAT ATG ACT TCC AAG CTG GCC G-3′ and antisense 5′-AAT GGA TCC TTA TGA ATT CTC AGC CCT C-3′; G3PDH sense 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ and antisense 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′. Cycling conditions were as follows: denaturing at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min for 25 cycles for G3PDH; denaturing at 95 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min for 35 cycles for COX-2 and IL-8.

Immunoblotting

Protein for COX-2 immunoblotting was collected in 1X PBS (pH 7.4), 5 mM EDTA (pH 8.0), 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin and incubated on ice for 10 min. Cells were sonicated at 30% power for 30 s. Protein concentrations were measured with the BioRad-DC kit (Hercules, CA, USA). Proteins (20 μg) were separated on a 10% Tris-glycine gel (Novex, San Diego, CA, USA) and transferred to an activated nylon membrane (PVDF-Plus, Micron Separations, Westborough, MA, USA). Equal protein

loading was confirmed by staining with Ponceau S red (Sigma, Milwaukee, WI, USA). Membranes were blocked with 3% (weight/volume) Carnation nonfat dry milk in Tris-buffered saline (TBST, 150 mM NaCl, 20 mM Tris HCl, pH 7.6, and 0.1% TWEEN-20). Rabbit polyclonal antibody to human COX-2 (Cayman Chemical, Ann Arbor, MI, USA) was added in fresh blocking solution (1 : 1000) and incubated for 1 h at room temperature. Membranes were washed three times in TBST, incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit immunoglobulin diluted 1 : 5000 in TBST, then washed as above. The enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL, USA) was used for detection. Membranes were stripped and re-probed with an antibody to β-actin (Sigma).

For analysis of phospho-ERK, phospho-p38 MAPK, phospho-JNK and unphosphorylated MAPK, cells were harvested in 50 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM EGTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 1% Triton X-100, 10% glycerol, 0.5% deoxycholic acid, 0.1% SDS, 50 mM β-glycerophosphate, 3 mM sodium vanadate (added fresh) and 10 µg/ml aprotinin (added fresh). Rabbit polyclonal anti-phospho-ERK1/2 (Promega, Madison, WI, USA), anti-phospho-p38 (Cell Signaling, Beverly, MA, USA), anti-phospho-JNK2 (Promega) antibodies directed against the unphosphorylated forms of ERK1/2, p38, and JNK (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at a dilution of 1 : 1000. Procedures were otherwise the same as described above.

Enzyme-linked immunoassay (EIA)

PGE_2 was measured by EIA using a kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Statistical analysis

Autoradiographs were scanned and analyzed using NIH Image. Statistical comparisons were made using Student's t-test.

Results

Ad/RSVLacZ infection rapidly increases expression of inflammatory mediators in RA synoviocytes

Increased expression of COX-2 mRNA and protein was observed after infection with Ad/RSVLacZ (Figures 1 and 2). Elevated COX-2 transcripts were seen by 1 h after infection, and were maximal by 4 h. COX-2 protein levels were increased by 4 h and reached maximal levels by 8 h after infection with Ad/RSVLacZ. The time-course of COX-2 up-regulation was similar to that seen after treatment with pro-inflammatory cytokines such as IL-1β [62]. In addition to COX-2, we evaluated induction of IL-8 mRNA. Similar to previous reports, IL-8 transcript levels were maximal by 8 h and returned to baseline overnight (data not shown) [6].

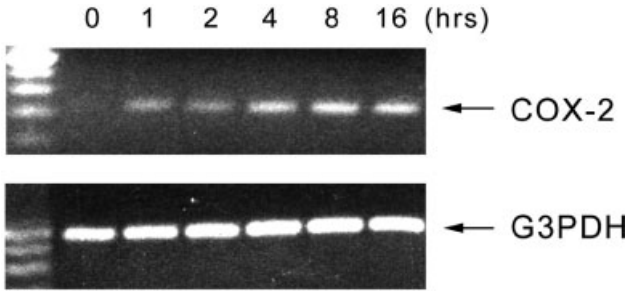

Figure 1. Ad/RSVLacZ infection of synoviocytes stimulates increased COX-2 mRNA. Synoviocytes were exposed to 10^4 infection-competent virus particles per cell and mRNA was harvested at the indicated times. RT-PCR demonstrates increased COX-2 mRNA by 1 h after virus exposure, reaches maximum levels by 4 h, and is sustained for at least 16 h. mRNA for G3PDH is unchanged by the treatment. The experiment was performed three times using three different primary cell lines isolated from three different patients yielding similar results

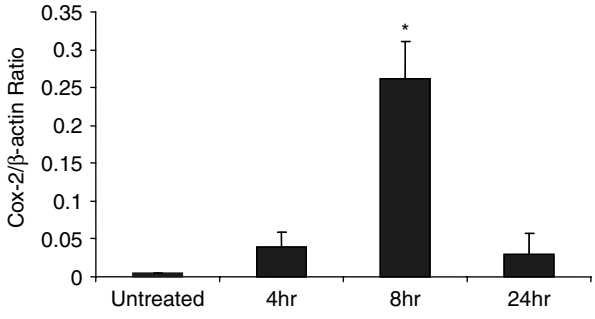

Figure 2. Ad/RSVLacZ infection of synoviocytes leads to up-regulated expression of COX-2 protein. Synoviocytes infected with 10^4 infection-competent virus particles per cell were cultured for variable times and the experiment was repeated three times using synoviocytes from three different patients. Immunoblotting was performed for COX-2, then membranes were reprobed for β-actin. COX-2 was normalized to β-actin and the relative expression is shown. We demonstrate a significant increase in levels of COX-2 protein maximal at 8 h post-exposure to Ad/RSVLacZ (*$P < 0.01$)

Transcription of adenoviral genes is not required for induction of inflammatory mediators

To determine if induction of inflammatory mediators was dependent on transcription of adenoviral genes, we compared induction of COX-2 by Ad/RSVLacZ before and after treatment with psoralen. Treatment with psoralen rendered the adenoviral DNA non-functional with respect to transcription while cell-surface binding and internalization were preserved. As shown in Figure 3, increased COX-2 protein was observed by 4 h and reached a similar maximal level of expression by 8 h in both untreated and psoralen-treated adenovirus. At 24 h,

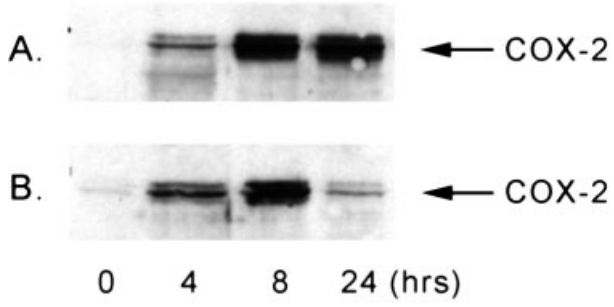

Figure 3. Transcriptional inactivation of Ad/RSVLacZ does not block early up-regulation of COX-2 expression. Synoviocytes were exposed to transcriptionally active adenovirus (A) or adenovirus inactivated by prior treatment with 8-methoxypsoralen and exposure to ultraviolet A band light (B). Increased COX-2 protein was seen by 4 and 8 h in both conditions. At 24 h, COX-2 expression was sustained only when active adenovirus were used for infection. Equal protein loading was confirmed by staining with Ponceau S red. The experiment was performed three times using primary cells isolated from three different patients cell lines which all yielded similar results

cultures treated with adenovirus not treated with psoralen demonstrated increased COX-2 protein. These results could reflect instability of the inactivated virus or that COX-2 expression may be dependent on transcription and expression of viral genes whereas early COX-2 expression requires only cell-surface binding and/or internalization.

Ad/RSVLacZ infection increases phosphorylation of MAPK

We evaluated potential signal transduction pathways for activation by Ad/RSVLacZ. We found that ERK and JNK were rapidly phosphorylated in RA synoviocytes after exposure to Ad/RSVLacZ, with increases that were statistically significant: $P < 0.005$ and $P = 0.005$, respectively (Figure 4). P38 MAPK was also increased, but changes were not statistically significant. Maximum intensity was seen at 5–15 min after treatment.

Up-regulation of COX-2 is mediated by MAPK

Induction of COX-2 by Ad/RSVLacZ was blocked by pretreatment with PD98059, an inhibitor of the MAPKK MEK-1/2 which activates ERK, and SB203580, which blocks activity of p38 MAPK (Figure 5). COX-2 levels were reduced by 66% by PD98059, 60% by SB2-3580, and

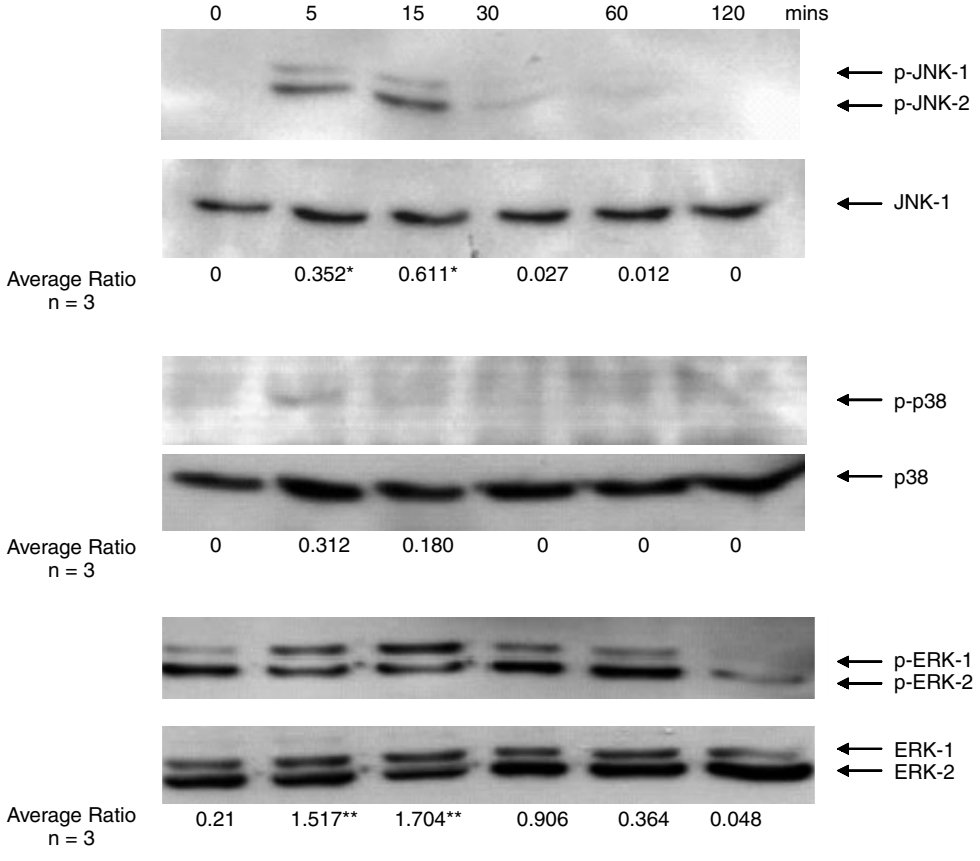

Figure 4. Adenovirus infection of synoviocytes increases phosphorylation of MAPK. Synoviocytes were infected with 10^4 infection-competent adenovirus particles per cell. Proteins were harvested at the indicated times. The phosphorylated forms of JNK, p38, and ERK were all transiently increased at 5–15 min after exposure to adenovirus, whereas there was no change in the unphosphorylated forms. The experiment was performed using primary cells isolated from three different patients with all experiments giving similar results. The ratio of phosphorylated/unphosphorylated forms were determined for each experiment. The average ratio for all three experiments is reported with significant changes for JNK and ERK (*$P = 0.05$, **$P < 0.05$)

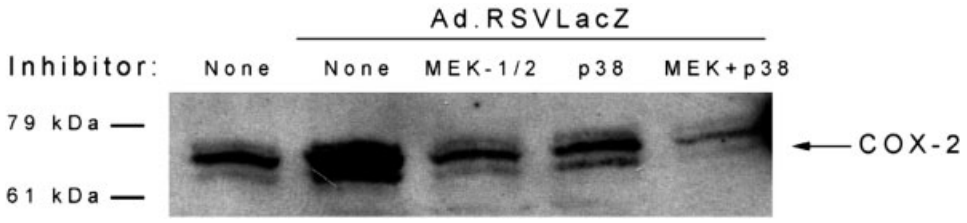

Figure 5. Up-regulation of COX-2 is blocked by inhibitors of MAPK. Synoviocytes were treated with adenovirus in the presence or absence of pre-treatment for 10 min with the MEK-1/2 inhibitor PD98059 (25 μM), the p38 MAPK inhibitor SB203580 (3 μM), or both. Proteins were harvested after 8 h and analyzed for COX-2 expression by immunoblotting. Both inhibitors blocked COX-2 up-regulation. Equal protein loading was confirmed by staining with Ponceau S red. The experiment was performed three times using primary synovial cells from three different patients with similar results

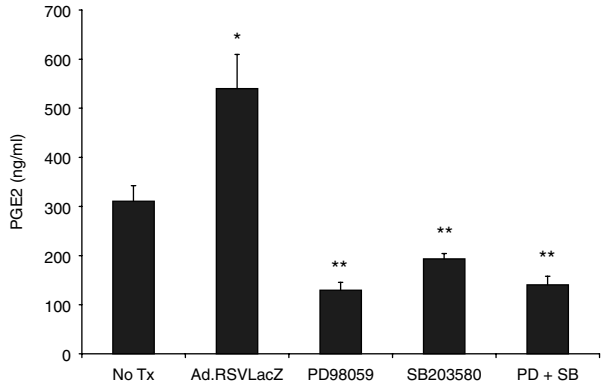

Figure 6. Inhibitors of MAPK block the adenovirus-stimulated increase of PGE_2. Synoviocytes were exposed to adenovirus in the presence or absence of the MEK-1/2 inhibitor PD98059 (25 μM), the p38 MAPK inhibitor SB203580 (3 μM), or both, applied 10 min prior to treatment. Adenovirus exposure significantly increased PGE_2 levels (*$P < 0.001$). Induced PGE_2 levels were reduced below baseline by either PD98059 ($P < 0.0001$), SB203580 (**$P < 0.0001$), or both (**$P < 0.0001$). There was no additive effect for the combination. The results represent two experiments each tested for PGE_2 in duplicate**

70% by the combination. In parallel with decreased COX-2 protein levels, PGE_2 production was also significantly reduced by pre-treatment with PD98059 and SB20358 ($P < 0.0001$) (Figure 6). These data indicate that up-regulation of COX-2 and increased PGE_2 are dependent, at least in part, on activation of MAPK.

Discussion

We demonstrated that Ad/RSVLacZ treatment of RA synoviocytes induces expression of COX-2 and subsequent generation of the key inflammatory mediator PGE_2. Because similar early patterns of COX-2 induction were observed with the administration of adenovirus-containing transcriptionally inactivated genomes to synoviocytes, we conclude that early induction of COX-2 does not require transcription of adenoviral genes. Adenoviral entry into cells is mediated by signaling pathways activated by binding the α_v integrins and requires MAPK pathway activation. Viral binding to synoviocytes results in activation of the MAPK family members, p42 ERK-1, p44 ERK-2, p38 MAPK, and JNK2. We speculate that binding of adenovirus to the α_v integrins is the critical factor for activation of the MAPKs that up-regulated COX-2. Preservation of the MAPK signaling pathways was necessary for activation of COX-2, and blockade of either the ERK or p38 pathways results in markedly diminished COX-2 expression and PGE_2 production. Similar to other stimuli for MAPK activation, stimulation by adenovirus binding occurs very rapidly but has sustained effects. It is likely that the MAPK cascade is important for production of a critical cellular protein and that blockade of the early signaling cascade is sufficient to block the program involved in generation of COX-2.

It has previously been reported by Hirschowitz *et al*. that a first-generation (ΔE1, ΔE3) adenoviral vector encoding a green fluorescent protein (GFP), prostate-specific antigen, or and a null vector induces COX-2 and PGE_2 in non-small-cell lung cancer cells [63]. These cells constitutively express COX-2 and relatively high levels of PGE_2. High levels of COX-2 in some malignant cells are thought important for cellular invasion, induction of angiogenesis, altered apoptosis, and increased immunologic resistance [64]. Hirschowitz and colleagues reported that the COX-2 protein was induced at 48 h post-infection time points and that blockade of ERK prevented the increase in PGE_2. In contrast to our results, they found that UV/psoralen-inactivated adenoviral vector did not increase PGE_2 levels. Differences between those data and the present report include differences in vector, the cell type examined, and the time points examined. Of interest, Zhang *et al*. reported that GFP itself can induce COX-2 and PGE_2. Those authors reported that the increase in COX-2 and PGE_2 is mediated by up-regulation of HSP70 and independent of MAPK and phosphatidylinositol-3-kinase signaling cascades [65]. The increase in PGE_2 was sufficient to induce vasodilation.

The synoviocytes used in these experiments were derived from patients with rheumatoid arthritis. Although many of the characteristics of these cells are controlled by the *in vivo* milieu, these cells do retain some phenotypic characteristics in culture. For example, rheumatoid synoviocytes exhibit anchorage-independent growth in early passage and can destroy articular cartilage [66,67]. In synoviocytes, COX-2 expression is stimulated by pro-inflammatory cytokines including IL-1β

and TNF-α [62,68–70]. The mechanisms leading to increased COX-2 expression by IL-1 include activation of NF-κB [62,71], translocation of c/EBP [72], and activation of MAPK-dependent transcription factors. The data presented here suggest that infection with an adenoviral vector can also increase expression of COX-2. We showed that up-regulated COX-2 expression was associated with increased PGE_2 production after exposure to Ad/RSVLacZ *in vitro*. In support of these findings, Caromody *et al*. demonstrated an increase in COX-2 staining in calvarial tissues that was enhanced in animals receiving AdLacZ [73]. Of interest, AdLacZ also stimulated osteoclastogenesis in their experiments, known to be enhanced in the presence of PGE_2.

In vivo, PGE_2 induces vasodilation, plasma extravasation, and sensitization of nociceptors [58]. In the context of adenovirus-based gene therapy administered locally for treatment of arthritis, stimulation of this pathway may not be of major clinical significance although it is difficult to predict the effects on joint tissues. However, similar regulatory pathways may be operative in vascular endothelial cells where regulation of COX-2 expression is similar to synovial cells. After intravascular administration, increased COX-2 expression and prostaglandin production may alter vascular tone and promote vascular leak syndromes.

Several gene therapy strategies have been employed using adenoviral-vector-based delivery of prostaglandin biosynthetic enzymes *in vivo*. The COX-1 gene alone or in a bicistronic construct with prostacyclin synthase resulted in augmented COX-1 and prostacyclin levels and reduced cerebrovascular infarct after intraventricular infusion [74]. They were unable to demonstrate increased COX-2 expression after adenovirus treatment. Another report demonstrated that adenovirally mediated expression of the inducible nitric oxide synthase proceeds by increased COX-2 as an obligatory downstream effector of cardioprotection after ischemia-reperfusion injury [75]. In that study, Ad5/LacZ control vector did not result in increased prostaglandin production in myocardial tissues, though it was unclear if there was an increase in COX-2 protein compared to an uninfected control.

IL-8 is a chemokine associated with infiltration of neutrophils and stimulation of angiogenesis in RA synovial tissues [76,77]. Previous data demonstrated increased IL-8 mRNA expression by active and inactive AdLacZ by 20 min after infection in HeLa cells [6]. We confirmed those data in primary human synoviocytes. In HeLa cells, IL-8 was not affected by cyclohexamide or heat inactivation of the virus, suggesting a direct effect of adenovirus binding. Furthermore, blocking activation of the Raf/MAPK pathway with forskolin resulted in inhibition of IL-8 production. Up-regulation of IL-8 and other cytokines and chemokines could contribute significantly to the net inflammatory response to adenovirus infection [6].

It was also shown that infection with adenovirus stimulates translocation of NF-κB to the nucleus [78,79]. Activation of NF-κB could act to stimulate expression of pro-inflammatory genes with NF-κB-responsive elements in their promoter regions [79]. A number of viruses activate NF-κB and have evolved mechanisms to utilize various properties of NF-κB to facilitate gene expression, replication, and evasion of immune responses [80,81]. Biphasic expression of NF-κB in the liver was seen after infusion of adenovirus in mice [78]. Early expression is not likely related to expression of adenoviral genes since activation occurred within 15 min after adenoviral infusion and was seen when viral E1, E2, E3 and L1-L4 regions were deleted [78]. However, later expression occurring 3 days after infusion is likely dependent on adenoviral genes since deletion of the above viral regions resulted in loss of NF-κB and expression of cytokines such as TNF-α [78]. We cannot exclude activation of NF-κB as a contributing factor to up-regulation of COX-2 in synovial cells following adenoviral infection [62]. Hirschowitz *et al*. found that NF-κB activity was not required for increased PGE_2 levels in non-small-cell lung cancer cells [63].

Understanding the nature of the inflammatory reaction triggered directly by the process of adenovirus infection has important implications for the design and utilization of adenoviral-based vectors for human gene therapy. In addition to the possible use of recombinant adenovirus as a local or regional drug delivery system, a better understanding of the immune inflammatory activation phenomenon associated with contact of adenovirus with synovial cells may provide insight into synoviocyte-mediated events that may be involved in triggering viral-associated arthritis or other reactive arthridities.

Acknowledgements

This work was supported by Public Health Service grant R21 AR46985 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases.

References

1. Baragi VM, Renkiewicz RR, Jordan H, *et al*. Transplantation of transduced chondrocytes protects articular cartilage from interleukin 1-induced extracellular matrix degradation. *J Clin Invest* 1995; **96**: 2454–2460.
2. Baragi VM, Renkiewicz RR, Qiu L, *et al*. Transplantation of adenovirally transduced allogeneic chondrocytes into articular cartilage defects in vivo. *Osteoarthritis Cartilage* 1997; **5**: 275–282.
3. Gerich T, Kang R, Fu F, *et al*. Gene transfer to the rabbit patellar tendon: potential for genetic enhancement of tendon and ligament healing. *Gene Ther* 1996; **3**: 1089–1093.
4. Goto H, Shuler F, Lamsam C, *et al*. Transfer of lacZ marker gene to the meniscus. *J Bone Joint Surg Am* 1999; **81**: 918–925.
5. Roessler BJ, Allen ED, Wilson JM, *et al*. Adenoviral-mediated gene transfer to rabbit synovium in vivo. *J Clin Invest* 1993; **92**: 1085–1092.
6. Bruder JT, Kovesdi I. Adenovirus infection stimulates the Raf/MAPK signaling pathway and induces interleukin-8 expression. *J Virol* 1997; **71**: 398–404.
7. Kozarsky KF, Wilson JM. Gene therapy: adenovirus vectors. *Curr Opin Genet Dev* 1993; **3**: 499.
8. Evans CH, Ghivizzani SC, Kang R, *et al*. Gene therapy for rheumatic diseases. *Arthritis Rheum* 1999; **42**: 1–16.

9. Apparailly F, Verwaerde C, Jacquet C, *et al*. Adenovirus-mediated transfer of viral IL-10 gene inhibits murine collagen-induced arthritis. *J Immunol* 1998; **160**: 5213–5220.
10. Ghivizzani SC, Lechman ER, Kang R, *et al*. Direct adenovirus-mediated gene transfer of interleukin 1 and tumor necrosis factor alpha soluble receptors to rabbit knees with experimental arthritis has local and distal anti-arthritic effects. *Proc Natl Acad Sci U S A* 1998; **95**: 4613–4618.
11. Goto H, Shuler F, Niyibizi C, *et al*. Gene therapy for meniscul injury: enhanced synthesis of proteoglycan and collagen by meniscal cells transduced with a TGFbeta(1)gene. *Osteoarthritis Cartilage* 2000; **8**: 266–271.
12. Lechman ER, Jaffurs D, Ghivizzani SC, *et al*. Direct adenoviral gene transfer of viral IL-10 to rabbit knees with experimental arthritis ameliorates disease in both injected and contralateral control knees. *J Immunol* 1999; **163**: 2202–2208.
13. Lubberts E, Joosten LA, van Den Bersselaar L, *et al*. Adenoviral vector-mediated overexpression of the IL-4 in the knee joint of mice with collagen-induced arthritis prevents cartilage destruction. *J Immunol* 1999; **163**: 4546–4556.
14. Ma Y, Thornton S, Duwel LE, *et al*. Inhibition of collagen-induced arthritis in mice by viral IL-10 gene transfer. *J Immunol* 1998; **161**: 1516.
15. Nixon A, Brower-Toland B, Bent S, *et al*. Insulin-like growth factor-I gene therapy applications for cartilage repair. *Clin Orthop* 2000; **379**: S201–S213.
16. Balter M. Gene therapy on trial. *Science* 2000; **288**: 951–957.
17. Gahery-Segard H, Juillard V, Gaston J, *et al*. Humoral immune response to the capsid components of recombinant adenoviruses: routes of immunization modulate virus-induced lg subclass shifts. *Eur J Immunol* 1997; **27**: 653–659.
18. Brody SL, Metzger M, Danel C, *et al*. Acute responses of non-human primates to airway delivery of an adenovirus vector containing the human cystic fibrosis transmembrane conductance regulator cDNA. *Hum Gene Ther* 1994; **5**: 821–836.
19. Christ M, Louis B, Stoeckel F, *et al*. Modulation of the inflammatory properties and hepatotoxicity of recombinant adenovirus vectors by the viral E4 gene products. *Hum Gene Ther* 2000; **11**: 415–427.
20. Juillard V, Villefroy P, Godfrin D, *et al*. Long-term humoral and cellular immunity induced by a single immunization with replication-defective adenovirus recombinant vector. *Eur J Immunol* 1995; **25**: 3467–3673.
21. Kafri T, Morgan D, Krahl T, *et al*. Cellular immune response to adenoviral vector infected cells does not require de novo viral gene expression: Implications for gene therapy. *Proc Natl Acad Sci U S A* 1998; **95**: 11 377–11 382.
22. McCoy RD, Davidson BL, Roessler BJ, *et al*. Pulmonary inflammation induced by incomplete or inactivated adenoviral particles. *Hum Gene Ther* 1995; **6**: 1553–1560.
23. Morral N, O'Neal W, Zhou H, *et al*. Immune responses to reporter proteins and high viral dose limit duration of expression with adenoviral vectors: comparison of E2a wild type and E2a deleted vectors. *Hum Gene Ther* 1997; **8**: 1275–1286.
24. O'Neal WK, Zhou H, Morral N, *et al*. Toxicological comparison of E2a-deleted and first-generation adenoviral vectors expressing alpha1-antitrypsin after systemic delivery. *Hum Gene Ther* 1998; **9**: 1587–1598.
25. Tripathy SK, Black HB, Goldwasser E, *et al*. Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. *Nat Med* 1996; **2**: 545–550.
26. Yang Y, Ertl HD, Wilson JM. MHC class I restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. *J Immunity* 1994; **1**: 433–442.
27. Yang Y, Nunes FA, Berencsi K, *et al*. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci U S A* 1994; **91**: 4407–4411.
28. Bergelson JM, Cunningham JA, Droguett G, *et al*. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* 1997; **275**: 1320–1323.
29. Tomko RP, Xu R, Philipson L. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxcackieviruses. *Proc Natl Acad Sci U S A* 1997; **94**: 3352–3356.
30. Hemmi S, Geertsen R, Mazzacasa A, *et al*. The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures. *Hum Gene Ther* 1998; **9**: 2363–2373.
31. Conget PA, Minguell JJ. Adenoviral-mediated gene transfer into ex vivo expanded human bone marrow mesenchymal progenitor cells. *Exp Hematol* 2000; **28**: 382–390.
32. Asaoka K, Tada M, Sawamura Y, *et al*. Dependence of efficient adenoviral gene delivery in malignant glioma cells on the expression levels of the coxsackievirus and adenovirus receptor. *J Neurosurg* 2000; **92**: 1002–1008.
33. Wang X, Bergelson JM. Coxsackievirus and adenovirus receptor cytoplasmic and transmembrane domains are not essential for coxsackievirus and adenovirus infection. *J Virol* 1999; **73**: 2559–2562.
34. Wickham TJ, Mathias P, Cheresh DA, *et al*. Integrins avb3 and avb5 promote adenovirus internalization but not virus attachment. *Cell* 1993; **73**: 309–319.
35. Goldman MJ, Wilson JM. Expression of avb5 integrin is necessary for efficient adenovirus-mediated gene transfer in the human airway. *J Virol* 1995; **69**: 5951–5958.
36. Delporte C, Redman RS, Baum BJ. Relationship between the cellular distribution of the alpha (v)beta3/5 integrins and adenoviral infection in salivary glands. *Lab Invest* 1997; **77**: 167–173.
37. Hashimoto Y, Kohri K, Akita H, *et al*. Efficient transfer of genes into senescent cells by adenovirus vectors via highly expressed alpha v beta 5 integrin. *Biochem Biophys Res Commun* 1997; **240**: 88–92.
38. Takayama K, Ueno H, Pei XH, *et al*. The levels of integrin alpha v beta 5 may predict the susceptibility to adenovirus-mediated gene transfer in human lung cancer cells. *Gene Ther* 1998; **5**: 361–368.
39. Tatebe S, Matsuura T, Endo K, *et al*. Adenoviral transduction efficiency partly correlates with expression levels of integrin alphavbeta5, but not alphavbeta3 in human gastric carcinoma. *Int J Mol Med* 1998; **2**: 61–64.
40. Byzova TV, Goldman CK, Pampori N, *et al*. A mechanism for modulation of cellular responses to VEGF: activation of the integrins. *Mol Cell* 2000; **6**: 851–860.
41. Li E, Stupack D, Klemke R, *et al*. Adenovirus endocytosis via av integrins requires phosphinositide-3-OH kinase. *J Virol* 1997; **72**: 2055–2061.
42. Li E, Stupack D, Bokoch GM, *et al*. Adenovirus endocytosis requires actin cytoskeleton reorganization mediated by Rho family GTPases. *J Virol* 1998; **72**: 8806–8812.
43. Li E, Stupack DG, Brown SL, *et al*. Association of p130cas with phosphatidylinositol-3-OH kinase mediates adenovirus cell entry. *J Biol Chem* 2000; **275**: 14 729–14 735.
44. Suomalainen M, Nakano MY, Boucke K, *et al*. Adenovirus-activated PKA and p38/MAPK pathways boost microtubule-mediated nuclear targeting of virus. *EMBO J* 2001; **20**: 1310–1319.
45. Yang S-H, Sharrocks AD, Whitmarsh AJ. Transcriptional regulation by the MAP kinase signaling cascades. *Gene* 2003; **320**: 3–21.
46. Subbaramaiah K, Marmo TP, Dixon DA, *et al*. Regulation of cyclooxgenase-2 mRNA stability by taxanes: evidence for involvement of p38, MAPKAPK-2, and HuR. *J Biol Chem* 2003; **278**: 37 637–37 647.
47. Paik J, Lee JY, Hwang D. Signaling pathways for TNFa-induced COX-2 expression: mediation through MAP kinases and NFkB, and inhibition by certain nonsteroidal anti-inflammatory drugs. *Adv Exp Med Biol* 2002; **507**: 503–508.
48. Huh YH, Kim SH, Kim SJ, *et al*. Differentiation status-dependent regulation of cyclooxygenase-2 expression and prostaglandin E2 production by epidermal growth factor via mitogen-activated protein kinase in articular chondrocytes. *J Biol Chem* 2003; **278**: 9691–9697.
49. Bradbury DA, Corbett L, Knox AJ. PI 3-kinase and MAP kinase regulate bradykinin induced prostaglandin E(2) release in human pulmonary artery by modulating COX-2 activity. *FEBS Lett* 2004; **560**: 30–34.
50. Mifflin RC, Saada JI, Di Mari JF, *et al*. Aspirin-mediated COX-2 transcript stabilization via sustained p38 activation in human intestinal myofibroblasts. *Mol Pharmacol* 2004; **65**: 470–478.
51. Mizumura K, Hashimoto S, Maruoka S, *et al*. Role of mitogen-activated protein kinases in influenza virus induction of prostaglandin E2 from arachidonic acid in bronchial epithelial cells. *Clin Exp Allergy* 2003; **33**: 1244–1251.

52. Dean JL, Sarsfield SJ, Tsounakou E, *et al*. p38 mitogen-activated protein kinase stabilizes mRNAs that contain cyclooxygenase-2 and tumor necrosis factor AU-rich elements by inhibiting deadenylation. *J Biol Chem* 2003; **278**: 39 470–39 476.
53. Crofford LJ. Prostaglandin biology. *Gastroenterol Clin NA* 2001; **30**: 863–876.
54. Stichtenoth DO, Thoren S, Bian H, *et al*. Microsomal prostaglandin E synthase is regulated by pro-inflammatory cytokines and glucocorticoids in primary rheumatoid synovial cells. *J Immunol* 2001; **167**: 469–474.
55. Narumiya S, FitzGerald GA. Genetic and pharmacological analysis of prostanoid receptor function. *J Clin Invest* 2001; **108**: 25–30.
56. Vane JR, Botting RM. Anti-inflammatory drugs and their mechanism of action. *Inflamm Res* 1997; **47**(Suppl 2): S78–S87.
57. Rocca B, FitzGerald GA. Cyclooxygenases and prostaglandins: shaping up the immune response. *Int Immunopharmacol* 2002; **2**: 603–630.
58. Crofford LJ. COX-1 and COX-2 tissue expression: Iimplications and predictions. *J Rheumatol* 1997; **24**(suppl 49): 15–19.
59. Hurwitz DR, Chinnadurai G. Evidence that a second tumor antigen coded by adenovirus early gene region E1a is required for efficient cell transformation. *Proc Natl Acad Sci U S A* 1985; **82**: 163–167.
60. Graham FL, van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 1988; **52**: 456–467.
61. Graham FL, Smiley J, Russell WC, *et al*. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 1977; **36**: 59–74.
62. Crofford LJ, Tan B, McCarthy CJ, *et al*. NF-κB in involved in the regulation of cyclooxygenase-2 expression by interleukin-1ß in rheumatoid synoviocytes. *Arthritis Rheum* 1997; **40**: 226–236.
63. Hirschowitz E, Hidalgo G, Doherty D. Induction of cyclo-oxygenase-2 in non-small cell lung cancer cells by infection with Δ E1, Δ E3 recombinant adenovirus vectors. *Gene Ther* 2002; **9**: 81–84.
64. Hawk ET, Viner JL, Dannenberg A, *et al*. COX-2 in cancer – a player that's defining the rules. *J Natl Cancer Inst* 2002; **94**: 545–546.
65. Zhang F, Hackett NR, Lam G, *et al*. Green fluorescent protein selectively induces HSP70-mediated up-regulation of COX-2 expression in endothelial cells. *Blood* 2003; **102**: 2115–2121.
66. Lafyatis R, Remmers EF, Roberts AB, *et al*. Anchorage-independent growth of synoviocytes from arthritic and normal joints: stimulation by exogenous platelet derived growth factor and inhibition by transforming growth factor-beta and retinoids. *J Clin Invest* 1989; **83**: 1267–1276.
67. Neidhart M, Seemayer CA, Hummel KM, *et al*. Functional characterization of adherent synovial fluid cells in rheumatoid arthritis: destructive potential in vitro and in vivo. *Arthritis Rheum* 2003; **48**: 1873–1880.
68. Crofford LJ, Wilder RL, Ristimaki AP, *et al*. Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues: effects of interleukin-1β, phorbol ester, and corticosteroids. *J Clin Invest* 1994; **93**: 1095–1101.
69. Hulkower KI, Wertheimer SJ, Levin W, *et al*. Interleukin-1β induces cytosolic phospholipase A2 and prostaglandin H synthase in rheumatoid synovial fibroblasts. Evidence for their roles in the production of prostaglandin E2. *Arthritis Rheum* 1994; **37**: 653–661.
70. Alaaeddine N, Di Battista JA, Pelletier JP, *et al*. Inhibition of tumor necrosis factor alpha-induced prostaglandin E2 production by the antiinflammatory cytokines interleukin-4, interleukin-10, and interleukin-13 in osteoarthritic synovial fibroblasts: distinct targeting in the signaling pathways. *Arthritis Rheum* 1999; **42**: 710–718.
71. Roshak AK, Jakcson JR, McGough K, *et al*. Manipulation of distinct NFκB proteins alters interleukin-1β-induced human rheumatoid synovial fibroblast prostaglandin E2 formation. *J Biol Chem* 1996; **271**: 31 496–31 501.
72. Thomas B, Crofford L, Humbert L, *et al*. Critical role of C/EBPδ and C/EBPβ factors in the stimulation of the cyclooxygenase-2 gene transcription by interleukin-1β in articular chondrocytes. *Eur J Biochem* 2000; **267**: 6798–6809.
73. Carmody EE, Schwarz EM, Puzas JE, *et al*. Viral interleukin-10 gene inhibition of inflammation, osteoclastogenesis, and bone resorption in response to titanium particles. *Arthritis Rheum* 2002; **46**: 1298–1308.
74. Lin HP, Lin TN, Cheung WM, *et al*. Cycloooxygenase-1 and bicistronic cyclooxygenase-1/prostacyclin synthase gene transfer protect against ischemic cerebral infarction. *Circulation* 2002; **105**: 1962–1969.
75. Li Q, Guo Y, Xuan YT, *et al*. Gene therapy with inducible nitric oxide synthase protects against myocardial infarction via a cyclooxygenase-2-dependent mechanism. *Circ Res* 2003; **92**: 741–748.
76. Szekanecz Z, Strieter RM, Kunkel SL, *et al*. Chemokines in rheumatoid arthritis. *Springer Sem Immunopathol* 1998; **20**: 115–132.
77. Belperio JA, Keane MP, Arenberg DA, *et al*. CXC chemokines in angiogenesis. *J Leukocyte Biol* 2000; **68**: 1–8.
78. Lieber A, He C-Y, Meuse L, *et al*. The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. *J Virol* 1997; **71**: 8798–8807.
79. Clesham GJ, Adam PJ, Proudfoot D, *et al*. High adenoviral loads stimulate NFkB-dependent gene expression in human vascular smooth muscle cells. *Gene Ther* 1998; **5**: 174–180.
80. Mosialos G. The role of rel/NF-kB proteins in viral oncogenesis and the regulation of viral transcription. *Cancer Biol* 1997; **8**: 121–129.
81. Lieber A, He CY, Meuse L, *et al*. Inhibition of NF-kappaB activation in combination with bcl-2 expression allows for persistence of first-generation adenovirus. *J Virol* 1998; **72**: 9267–9277.

