Shear-Induced Cyclooxygenase-2 via a JNK2/c-jun-Dependent Pathway Regulates Prostaglandin-Receptor Expression in Chondrocytic Cells

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Abbreviations: COX, cyclooxygenase; IL-1, interleukin-1; AP-1, activator protein-1; CRE, cAMP-response element; PGE2, prostaglandin E2; JNK, c-jun N-terminal kinase; RPA, ribonuclease protection assay; TMEV, TIGR Multi Experiment Viewer; NSAIDs, nonsteroidal anti-inflammatory drugs; MKK, MAP kinase kinase

Running Title: Shear-Induced COX-2 Regulation in Chondrocytic Cells

Keywords: shear, chondrocyte, microarray, cyclooxygenase, prostaglandin receptors
ABSTRACT
Using cDNA microarrays coupled with bioinformatics tools, we elucidated a signaling cascade regulating cyclooxygenase-2 (COX-2), a pivotal pro-inflammatory enzyme expressed in rheumatic and osteoarthritic, but not normal, cartilage. Exposure of T/C-28a2 chondrocytic cells to fluid shear results in co-regulation of c-jun N-terminal kinase2 (JNK2), c-jun and COX-2 as well as concomitant downstream expression of prostaglandin receptors EP2 and EP3a1. JNK2 transcript inhibition abrogated shear-induced COX-2, EP2 and EP3a1 mRNA upregulation, as well as c-jun phosphorylation. Functional knock-out experiments using an antisense c-jun oligonucleotide revealed the abolition of shear-induced COX-2, EP2 and EP3a1, but not JNK2, transcripts. Moreover, inhibition of COX-2 activity eliminated mRNA upregulation of EP2 and EP3a1 induced by shear. Hence, a biochemical pathway exists wherein fluid shear activates COX-2, via a JNK2/c-jun-dependent pathway, which in turn elicits downstream EP2 and EP3a1 mRNA synthesis.
INTRODUCTION

Cyclooxygenase (COX) is a critical pro-inflammatory enzyme that converts arachidonic acid to prostaglandins which have been implicated in the pain and inflammation of rheumatic disease (1). COX is known to exist in two isoforms, namely COX-1 and COX-2, with similar sequence identity (1, 2). Although the two isoforms have analogous active site structures, catalytic mechanisms, products and kinetics, they exhibit differences in their regulation and function (1). COX-1, is constitutively expressed in many tissues and cell types, and is presumed to be responsible for the synthesis of “housekeeping” prostaglandins that are critical for normal physiological functions (1). However, COX-1 has been reported to be differentially regulated in endothelial cells stimulated with fluid shear (3) or phorbol 12-myristate 13-acetate (4), as well as in mouse osteoblastic MC3T3 cells treated with basic fibroblastic growth factor (5). Nevertheless, a variety of chemical stimuli such as interleukin-1 alpha (IL-1α) have failed to regulate COX-1 in chondrocytic cells (6).

On the other hand, COX-2 is expressed in few tissues under basal conditions. However, COX-2 mRNA and protein synthesis are induced in a time- and dose- dependent manner in inflammation models such as cytokine-stimulated human macrophages (7) and rat mesangial cells (8). Moreover, in chondrocytic models, COX-2 is regulated by chemical agonists such as okadaic acid via Activator Protein-1 (AP-1) and cAMP-response element (CRE) binding proteins (9), as well as nitric oxide via extracellular signal-related protein kinase 1/2 and p38 kinase (10), and tumor necrosis alpha via NF-kB (11). Thus, several discrete signaling pathways have been implicated in the genesis of COX-2 synthesis which are dependent on the stimulus imposed on the cell. Although fluid shear has been reported to induce COX-2 expression in endothelial (3,
COX inhibitors have been extensively used in the treatment of rheumatoid arthritis. Of the various prostanoids, prostaglandin E₂ (PGE₂) is thought to play a key role in the erosion of cartilage and juxta-articular bone. The biological actions of PGE₂ are mediated through its binding to specific G-protein-coupled cell surface prostaglandin EP receptors. There are at least four subtypes of EP receptors, termed EP1, EP2, EP3, and EP4, that directly modulate intracellular levels of inositol phosphate or cAMP (16). The significance of the prostaglandin EP receptors was recently documented in a mouse model of experimentally induced arthritis (17). COX-1, but not COX-2, has been implicated in the regulation of the expression of the prostaglandin receptors in cervical carcinomas (18). On the other hand, inactivation of COX-2 has been reported to increase EP3 and EP4 receptor expression in a murine kidney cell line (19), although it failed to interfere with it in an osteogenic cell line (20). Nevertheless, the effects of mechanical stimuli and COX activity on the regulation of prostaglandin receptors in chondrocytic cells have yet to be examined.

Evidence suggests that abnormal mechanical loading of cartilage may be detrimental to the tissue. Pressure gradients generated from mechanical loading during daily activities drive interstitial fluid movement within the cartilage tissue, suggesting that fluid shear is a pathophysiologically relevant mechanical signal in cartilage metabolism. In this regard, our studies have been directed at examining the effects of shear flow on the regulation of COX-1 and COX-
2 expression in chondrocytic cells, as well as elucidating potential upstream and downstream pathways, using microarray technology and computational analysis in conjunction with traditional molecular biology techniques. Our findings show that high shear stress (20 dyn/cm²) activates the signaling molecule c-jun N-terminal kinase 2 (JNK2), which then triggers the phosphorylation of the transcription factor c-jun. These signaling events are involved in the shear-induced upregulation of COX-2 at the mRNA and protein levels. Given that COX-1 is not regulated by fluid shear, we show through the use of a specific COX-2 inhibitor that COX-2 activity ultimately stimulates the synthesis of the prostaglandin receptor subtypes EP2 and EP3a1.

**MATERIALS AND METHODS**

**CELL CULTURE**

The human T/C-28a2 chondrocytic cell line (21-23) was chosen as a model system for monitoring chondrocytic cellular responses to fluid shear for three important reasons. First, the T/C-28a2 cells have been extensively characterized, and shown to behave much like primary human chondrocytes when cultured under appropriate conditions (21-23). Second, a sufficient number of primary human chondrocytic cells to perform microarray and ribonuclease protection assay (RPA) experiments are difficult to obtain from a single operative procedure, whereas the chondrocytic phenotype is gradually lost in primary cultures over serial passaging. Finally, the inherent genetic variation in human chondrocyte cultures prepared from different cartilage donors could make reproducibility an issue. T/C-28a2 cells were cultured in a 1:1 mixture of Ham's F-12 and Dulbecco’s Modified Eagle Medium (DMEM) (Biowhitaker), supplemented with 1X Antibiotic-Antimycotic (Invitrogen) and 10% Fetal Bovine Serum (FBS) (Paragon
Biotech), and propagated for a maximum of ten passages. Prior to shearing experiments, T/C-28a2 cells were detached from tissue culture flasks by mild trypsinization, then seeded (1x10⁶ cells/ml) on 75x38 mm glass slides (Corning Glass), and incubated overnight at 37°C in 10% FBS-containing medium. 24 hr prior to shear stress exposure, T/C-28a2 cells were washed with Dulbecco’s Phosphate Buffered Saline lacking Ca²⁺/Mg²⁺ (DPBS), and incubated in serum-free medium composed of 1:1 Ham's F-12 and DMEM, supplemented with 1X Antibiotic-Antimycotic, and 1% Nutridoma SP (Roche) to maintain chondrocytic phenotype (23). Throughout cell culturing and shearing experiments, the T/C-28a2 cells maintained their polygonal morphology, and failed to exhibit Type I collagen expression, both of which are congruent with the chondrocyte phenotype (21).

**SHEAR STRESS EXPOSURE**

Confluent monolayers of T/C-28a2 cells were exposed to shear stress (4, 10, or 20 dyn/cm²) for pre-defined periods of time by using a parallel plate flow chamber placed in a flow loop gassed with 95% air and 5% CO₂, and enclosed within a 37°C convective air incubator (24, 25). Serum-free media containing 1% Nutridoma was circulating in the shear apparatus for the duration of the experiment. As a control, T/C-28a2 cells were placed on flow chambers, but no shear flow was generated. These matched static specimens were otherwise exposed to the same conditions as their shear analogs.

**RNA ISOLATION**

RNA, from sheared specimens and their matched static controls, was immediately isolated using Trizol reagent (Invitrogen). T/C-28a2 cells from four independent flow chambers were pooled
together to generate a sheared (or static) sample for either RPA or microarray analysis. RNA was quantified using UV spectrophotometry at the OD of 260nm, and stored at -80°C until assayed.

**RIBONUCLEASE PROTECTION ASSAY**

RPA was used to quantify the relative mRNA expression levels for a selected number of genes at the end of shear flow exposure as compared to static controls. Clones for c-fos, JNK2, c-jun, COX-1, COX-2, prostaglandin receptors EP3a1 and E2, and the housekeeping gene GAPDH (Research Genetics) were digested at appropriate restriction sites to serve as in-vitro transcription templates. Antisense RNA probes were generated using either T3 or T7 polymerase (Promega) in the presence of 32P-UTP (ICN Radiochemicals), and allowed to hybridize overnight at 55°C to 10 µg of total RNA isolated from the shear and static specimens. The RNA:RNA duplexes were digested with RNAses at 30°C for 1 hr (BD Pharmingen). RNAses was inactivated by incubating with Proteinase K (BD Pharmingen) at 37°C for 20 min, and extracted using phenol/chloroform/isoamyl alcohol (Invitrogen). The mixture was ethanol precipitated, and RNA fragments were resolved using a denaturing 5% monomer (19:1 Acrylamide/Bisacrylamide) gel (National Diagnostics). The gel was dried, exposed to film, and bands of interest were subjected to densitometric analysis.

**PROBE GENERATION AND PURIFICATION FOR MICROARRAY EXPERIMENTS**

10 µg of total RNA were reversed transcribed in a mixture containing 6 µg random hexamers (Invitrogen), 0.01M DTT (Invitrogen), 1X Aminoallyl-dNTP mixture (25mM each of dATP, dCTP, dGTP, 15mM of dTTP, and 10mM of Aminoallyl-dUTP) (Sigma), 1X Reaction Buffer,
and 400U SuperScript II Reverse Transcriptase (Invitrogen) at 42°C overnight. RNA template was then hydrolyzed by adding NaOH and EDTA to a final concentration of 0.2M and 0.1M, respectively, at 70°C for 15 min. Unincorporated Aminoallyl-dUTP was removed using a Qiagen QIAquick column (26). The probe was eluted using a phosphate elution buffer (4mM KPO₄ pH = 8.5 in ultrapure water), dried, and resuspended in 0.1M carbonate buffer (26). To couple the Aminoallyl with fluorescent labels, NHS-Cy3 (in static control samples) or NHS-Cy5 (in sheared specimens) (Amersham) were added in the dark at room temperature (RT) for 1 hr. Uncoupled label was removed using the QIAquick column (26).

MICROARRAY HYBRIDIZATION, NORMALIZATION AND ANALYSIS

Aminosilane-coated microscope slides printed with a set of 32,448 Expressed Sequence Tags were prehybridized in 5X SSC (Invitrogen), 0.1% SDS (Invitrogen), and 1% BSA (Sigma) at 42°C for 45 min (26). Subsequently, the slides were washed at RT with deionized water, dipped in 100% isopropanol at RT, and allowed to dry. Equal volumes of the Cy-3 and Cy-5 labeled probes were combined, and supplemented with 20 µg each of COT1-DNA and Poly(A)-DNA (26). This mixture was heated to 95°C for 3 min, after which an equal volume of hybridization buffer, composed of 50% formamide (Roche), 10X SSC and 0.2% SDS, was added. The probes were added to the microarray slide, and allowed to hybridize at 42°C overnight. Subsequently, the slide was sequentially washed in a solutions containing 1X SSC and 0.2% SDS at 42°C, 0.1X SSC and 0.2% SDS at RT, and 0.1X SSC at RT, each for 4 min (26), then air-dried and scanned using the ScanArray 3000 (GSI Lumonics).

Expression ratios from individual genes were extracted using TIGR Spotfinder, and normalized with the total intensity algorithm of TIGR Multi Experiment Viewer (TMEV) (26).
Three independent experiments were performed for each condition, thereby permitting an analysis that satisfies conservative statistical criteria. Genes with a ratio of measured Cy5 to Cy3 intensities of $\geq 2.0$ for each of the three experiments were considered positively regulated by shear stress, whereas those with a ratio $\leq 0.5$ were regarded negatively regulated (12).

Following data normalization, average linkage hierarchical clustering analysis with a Euclidean distance metric was performed using TMEV (26), in which genes are iteratively grouped together based on their distance metric. Alternatively, clustering analysis was performed using Self-Organizing Maps (SOMs, Euclidean distance metric, 3x3 Hexagonal Topology) (27), in which a neural network is trained via competitive learning and subsequently used to cluster the gene expression data. Both clustering algorithms were executed on a dataset containing elements that had a fluorescence value of at least 500, in order to eliminate points suffering from poor hybridization or spotting which may have potentially confounded the clustering output and interpretation.

**WESTERN HYBRIDIZATION**

T/C-28a2 cells, from sheared and matched static control specimens, were harvested by mild trypsinization, and washed with DPBS. Total protein was liberated using a cell lysis buffer (10mM NaPO$_4$, 2mM EDTA, 10mM NaN$_3$, 120mM NaCl, 1% Deoxycholate, 1% NP-40, and protease inhibitor) (28), separated by 10% SDS-PAGE, and electrotransferred on PVDF membrane (Millipore). The membrane was blocked overnight in 5% blocking solution (Biorad) at 4°C, incubated with a primary antibody against JNK2, COX-1, COX-2, c-jun, or phospho-c-jun (1:300; Santa Cruz Biotech) for 3 hr at RT, washed 5X in TBST, and incubated for 1 hr with
a goat anti-rabbit horseradish peroxidase secondary antibody (1:3000; Sigma) at RT. The membrane was washed 3X in TBST, and reactive bands were detected using a Super Signal chemiluminescent substrate kit (Pierce). To ensure equal loading of samples in each lane, membranes were stripped and re-probed with a β-actin antibody (1:300; Santa Cruz Biotech).

**INHIBITION STUDIES**

To inhibit viable JNK2, c-jun or c-fos transcript, an antisense oligonucleotide specific for JNK2, c-jun or c-fos (Isis Pharmaceuticals) was transfected into the T/C-28a2 cells. Briefly, confluent T/C-28a2 cells were washed, and incubated with 400nM of the c-fos, JNK2 or c-jun antisense oligonucleotide with lipofectin reagent (Invitrogen) in 1ml of serum-free medium. After 5 hr, 1 ml of medium supplemented with 2% Nutridoma was added. Cells remained in a 95% air and 5% CO₂ for 24 hr until they were exposed to shear flow or stationary conditions. β-galactosidase staining was used to verify transfection efficiency. The antisense oligonucleotide targeting JNK2 and a mismatch control oligonucleotide were synthesized as uniform phosphorothioate, chimeric oligonucleotides with 2’-O-methoxyethyl modified sugars on nucleotides 1-5 and 16-20 and 2-deoxy sugars on nucleotides 6-15. The antisense oligonucleotides targeting c-jun or c-fos alone and a chemistry control oligonucleotide were synthesized as uniform phosphorothioate oligonucleotides and 2’-deoxy sugars on all nucleotides. The oligonucleotides were synthesized using an Applied Biosystems 380B automated DNA synthesizer (Perkin Elmer - Applied Biosystems) and purified as described previously (29). The sequences of the oligonucleotides used in these studies are as follows: JNK2 antisense 5’-GCTCAGTGGACATGGATGAG-3’, JNK2 control 5’-GCACATTGCACGTGAATTAC-3’, c-jun antisense 5’-TCAGCCCCCGACGGTCTCTC-3’, c-
fos antisense 5’-AAGTCCTTGAGGCCACAGC-3’, c-jun/c-fos control 5’-GTGCAGCAGCCGAAATC-3’.

To inhibit COX-2 enzyme activity, the specific inhibitor NS-398 (Cayman Chemical), was used at a concentration of 30 µM (30) within the flow media for the entire duration of the shear experiment, as well as for their respective matched static control specimens.

**IMMUNOFLUORESCENCE ANALYSIS**

After 24 hr, T/C-28a2 cells on a glass slide from sheared specimens and their matched static controls were washed with DPBS, and fixed for 2 min either in ice cold pure acetone or methanol. Thereafter, specimens were washed in DPBS, and incubated with 10% normal blocking serum for 20 min. Cells were then incubated with COX-2 antibody (2 µg/ml; Oxford Biomedical) in 1.5% normal blocking serum for 1hr at 37°C, and subsequently washed 3X with DPBS 5 min each. A fluorescein conjugated secondary antibody was added for 45 min at RT, followed with three washes in DPBS. Samples were then mounted using anti-fade mounting medium, and examined using confocal microscopy.

**RESULTS**

To investigate chondrocytic cellular responses to fluid shear, T/C-28a2 chondrocytic cells were exposed to a stress level of 20 dyn/cm² (representative of a high shear environment as evident in previous studies (31-33)) for prescribed periods of time, using a parallel-plate perfusion chamber placed in a flow loop. Microarray analysis revealed that 301 known genes responded to shear stress after a 24-hr chondrocytic cell shear exposure, of which 188 were
upregulated, whereas 113 were downregulated as compared to matched static controls (Abulencia et al., manuscript in preparation). Shorter shear exposure times (1.5 hr) resulted in the upregulation and downregulation of 34 and 30 known genes, respectively, some of which are listed in Fig. 1. Using an average linkage hierarchical clustering algorithm with Euclidean distance metric (TMEV program), we determined that JNK2, c-jun and COX-2 are contained within the same sub-tree structure (supplemental figure 1a), thereby implicating co-regulation among the three genes upon T/C-28a2 cell exposure to fluid shear. Furthermore, this analysis revealed that the prostaglandin EP2 and EP3a1 receptors, which may act as potential downstream targets of the COX activity, are co-regulated, though residing in a sub-tree structure different from the aforementioned one (supplemental figure 1a). The gene expression data were also analyzed using SOMs (Euclidean distance metric, 3x3 Hexagonal Topology). The results from this analysis indicate that JNK2, c-jun, COX-2 and both prostaglandin EP2 and EP3a1 receptors are contained within the same cluster (supplemental figure 1b). Moreover, hierarchical analysis within the aforementioned cluster reveals the existence of distinct sub-tree structures in which JNK2, c-jun and COX-2 are tightly grouped together, separately from the prostaglandin EP2 and EP3a1 receptors (supplemental figure 1b). Cumulatively, these results indicate that the clustering pattern of the selected genes is conserved irrespective of the algorithm implemented. These microarray clustering data prompted us to hypothesize that fluid shear activates COX-2, via a JNK2-dependent pathway involving phosphorylation of c-jun, which in turn elicits downstream EP2 and EP3a1 mRNA synthesis.

As a first step, we examined the accuracy of the microarray gene expression profiling by performing an RPA analysis on the aforementioned genes. Fig. 1 shows microarray ratios, RPA
ratios, and RPA autoradiographs on these selected genes at both 1.5 and 24 hr. The results from
the RPA analysis are in very good agreement with the microarray data, thus validating our
microarray procedure and analysis. We next wished to systematically investigate whether
upregulation of JNK2, c-jun and COX-2 precedes that of EP2 and EP3a1. To this end, an RPA
time-course experiment was performed at 45 min, 1.5, 3, 6, 12, 16 and 24 hr. As shown in Fig.
2A, the mRNA expression levels of JNK2, c-jun and COX-2 increased concurrently after
exposure of chondrocytic cells to shear stress for 1.5 hr, and also remained elevated at the 24 hr
time point. In marked contrast, significant upregulation of EP2 and EP3a1 mRNA expression
occurred only after 24 hr of shear exposure (Fig. 2B), and ensued COX-2 protein expression
which was detected at the 16 hr time point (Fig. 3A). No COX-2 protein was detected at earlier
time points in both static and sheared samples (Fig. 3A). Subsequent experiments aimed to
determine the localization of COX-2 protein after the application of shear stress, using
immunofluorescence analysis. In accord with the Western blotting data, COX-2 was absent from
T/C-28a2 chondrocytic cells subjected to static (no-flow) conditions for 24 hr (Fig. 3B).
However, COX-2 staining was evident in chondrocytic cells sheared for 24 hr at 20 dyn/cm², and
observed to be present in the cytosol and nuclear envelope of shear-stimulated cells (Fig. 3B).

To demonstrate the involvement of the JNK2 pathway in the shear-induced regulation of
COX-2, an antisense oligonucleotide inhibiting the JNK2 transcript was transfected into T/C-
28a2 chondrocytic cells prior to their exposure to a shear stress level of 20 dyn/cm² for 24 hr.
The results indicate that JNK2 inhibition abrogated shear-induced COX-2 expression at both the
protein (Fig. 4A) and mRNA (Figs. 5A, B) levels. In distinct contrast, a control oligonucleotide
failed to affect shear-induced COX-2 expression (Fig. 4A). To verify the efficacy of the
antisense JNK2-specific oligonucleotide, Western immunoblot (Fig. 4B) and RPA (Figs. 5A, B) analyses were carried out showing that it abrogated shear-induced JNK2 protein and mRNA upregulation, while the control oligonucleotide had no effect (Fig. 4B). Cumulatively, these data suggest that the JNK2 represents an upstream signaling element of the COX-2 expression in response to fluid shear.

Among JNKs, JNK2 has been shown to exhibit the highest affinity for c-jun, and to possess a putative loop region that interacts with the JNK-docking site on c-jun (34). We therefore examined the effects of shear-induced JNK2 activity on c-jun phosphorylation in chondrocytic cells. Western blot analysis revealed that application of high shear stress (20 dyn/cm²) to T/C-28a2 cells induced phosphorylation of c-jun, which is abrogated by the presence of an antisense oligonucleotide inhibiting the JNK2 transcript, but not of a control oligonucleotide (Fig. 4B). To further examine the role of c-jun in this pathway, an antisense oligonucleotide directed against c-jun was transfected into T/C-28a2 chondrocytic cells prior to their exposure to shear (20 dyn/cm² for 24 hr). The results indicate that antisense oligonucleotide functional knockout of c-jun abrogated shear-induced COX-2 mRNA expression levels, while leaving intact the upstream JNK2 transcript (Figs. 5A, B). In distinct contrast, a control oligonucleotide failed to affect shear-induced COX-2 expression (data not shown).

Previous work on chondrocytic cells stimulated with okadaic acid suggested a role for the transcription factor c-fos in the regulation of COX-2 expression(9). We therefore wished to examine its involvement in the induction of COX-2 in chondrocytic cells subjected to shear. Microarray clustering analysis indicates that c-fos is not contained in the same sub-tree structure with JNK2, c-jun and COX-2, suggesting that it is not likely to be involved in this pathway.
was corroborated by functional knockout experiments using an antisense c-fos-specific oligonucleotide showing that it failed to affect shear-induced COX-2 expression (Figs. 5A, B). Evidence for the transfection efficiency and functionality of the antisense oligonucleotide to c-fos is provided by RPA analysis demonstrating that cells transiently transfected with the antisense oligomer, but not a control one, exhibited abolition of shear-induced c-fos upregulation (Figs. 5A, B). Taken altogether, we propose that shear-induced COX-2 expression is regulated by a JNK2-dependent pathway involving c-jun phosphorylation. It is noteworthy that inhibition of JNK2 or c-jun, but not c-fos, activity also abolishes EP2 and EP3a1 mRNA synthesis (Figs. 5A, B), providing support to our hypothesis that these prostaglandin receptor subtypes represent downstream targets of COX-2 activity. The effects of antisense oligonucleotides on the shear regulation of the selected genes of this study were also validated using the cDNA microarray technology (data not shown).

Previous work has shown that COX-1, rather than COX-2, is involved in the regulation of the prostaglandin receptors in cervical carcinomas (18). Moreover, there is evidence, albeit contradictory (3, 13, 14), suggesting that fluid shear regulates COX-1 expression in human vascular cells. As a first step, we examined the effects of high shear stress (20 dyn/cm²) on COX-1 regulation in T/C-28a2 chondrocytic cells. The results indicate that COX-1 expression is not altered by fluid shear at both RNA and protein levels (Figs. 4A and 5A, B). It is worth noting that inhibiting JNK2, c-jun or c-fos transcripts does not affect COX-1 expression in the sheared versus static specimens either (Figs. 5A, B). An RPA analysis of T/C-28a2 chondrocytic cells which were subjected to shear (20 dyn/cm² for 24 hr) in the presence or absence of the specific COX-2 inhibitor, NS-398, revealed that blockade of COX-2 activity
abolished EP2 and EP3a1 mRNA upregulation (Figs. 5A, B). These observations were also confirmed using cDNA microarrays (data not shown). Taken altogether, these data indicate that shear-induced COX-2 activity, but not COX-1, regulates EP2 and EP3a1 mRNA synthesis. This observation is in clear contrast to previous work showing that COX-2 inactivation upregulates prostaglandin EP3 and EP4 receptor expression in a murine kidney cell line (19).

Exposure of cartilage to abnormal mechanical loading may lead to cellular and biochemical changes that are associated with cartilage degradation and the progression of arthritis. Nonsteroidal anti-inflammatory drugs (NSAIDs) represent an effective therapy for treating arthritic diseases, and elicit their responses by interfering with COX activity. We therefore wished to determine whether exposure of T/C-28a2 chondrocytic cells to lower levels of shear would induce COX-2 expression. The results indicate that application of a wall shear stress of 4 dyn/cm² to T/C-28a2 chondrocytic cells for a duration of 24 hr did not significantly affect the gene transcript levels of the subset of genes examined in this work between the static and sheared samples (Fig. 5C). However, by increasing fluid shear to 10 dyn/cm², a significant upregulation (4.7 fold) of the JNK2 transcript was detected by an RPA analysis, while the transcript levels of the other genes in the subset remained relatively unchanged (Fig. 5C). These findings underscore the effect of shear stress magnitude on gene regulation in chondrocytic cells, and provide further support to concept that JNK2 activation is the first step in the proposed cascade of events, and necessary for the regulation of COX-2 activity as well as downstream targets.
DISCUSSION

By coupling cDNA microarray technology with bioinformatics tools, we proposed a novel signaling pathway regulating COX-2 expression in chondrocytic cells exposed to mechanical stimulation. The conservation of the clustering patterns using two distinct algorithms provided evidence for the likely existence of the proposed pathway, which was validated using traditional molecular biology techniques. In particular, we demonstrate that fluid shear activates the signaling molecule JNK2 which then triggers the phosphorylation of the transcription factor c-jun. These signaling events directly regulate COX-2 expression at both the mRNA and protein levels. Moreover, shear-induced COX-2 activity, but not COX-1, ultimately stimulates the synthesis of the prostaglandin receptor subtypes EP2 and EP3a1 in chondrocytic cells.

Previous studies on endothelial cells have shown that low levels of fluid shear (1-4 dyn/cm²) elicit COX-2 expression (12, 14). This finding is in clear contrast to the observations made in our study where induction of COX-2 was not detected after chondrocytic cell exposure to a shear stress level lower than 20 dyn/cm². The enhanced mechanosensitivity of human umbilical vein endothelial cells in comparison to chondrocytic cells may be related to the physiological shear environment (1-4 dyn/cm²) that these cells encounter in vivo. In contrast, chondrocytes reside in cartilage which functions to absorb mechanical loading that arises during daily activities. Since abnormal mechanical loading of cartilage may be detrimental to the tissue, expression of pro-inflammatory genes such as COX-2 in response to low levels of fluid shear would offer an unfavorable phenotype for chondrocytic cells. Hence, our data demonstrating the requirement of an elevated shear stress threshold for the induction of COX-2 expression suggest
that an abnormally high mechanical loading is necessary to potentially elicit COX-2-mediated inflammation and cartilage degradation within articular joints.

Analysis of the human COX-2 gene has revealed the presence of regulatory sites such as a TATA box, a C/EBP motif, two AP-2 sites, 3 SP-1 sites, two NF-κB sites, a CRE motif, and an Ets-1 site (9). Previous studies have demonstrated the critical involvement of the CRE binding site in the shear-induced transcription of COX-2 in MC3T3-E1 osteoblastic cells (15) and endothelial cells (14). Nevertheless, the transcription factors and signaling intermediates regulating COX-2 induction have not previously been determined. In our study, we provide solid evidence showing that c-jun regulates COX-2 transcription in T/C-28a2 chondrocytic cells stimulated with fluid shear. If CRE is indeed involved in the regulation of COX-2 in T/C-28a2 chondrocytic cells subjected to fluid shear, it is likely that c-jun forms heterodimers with either members of the ATF family or c-fos, which can bind to CRE sequences (34). However, microarray clustering analysis reveals that c-fos is not found in the same sub-tree structure with COX-2 and c-jun, suggesting that COX-2 regulation may be independent of c-fos signaling. This is further substantiated by our functional knockout experiments using a c-fos antisense oligonucleotide demonstrating that c-fos inhibition does not affect COX-2 expression. This finding is in marked contrast to results obtained with chondrocytes stimulated with okadaic acid wherein c-fos, JunB and possibly c-jun are involved in upstream regulatory binding (9). The aforementioned difference as well as the absence of junB and junD regulation in response to shear (data not shown) as opposed to okadaic acid stimulation underscore that discrete signaling pathways are implicated in COX-2 regulation which are dependent on the stimulus imposed on the cell.
Using antisense oligonucleotides directed against JNK2, we demonstrated its critical involvement in the biochemical pathway regulating shear-induced COX-2 expression in T/C-28a2 chondrocytic cells. More specifically, we showed that fluid shear activates JNK2 which in turn phosphorylates the transcription factor c-jun. It is now established that JNK activation occurs via either MAP kinase kinase 7 (MKK7) or MKK4 phosphorylation of JNK Thr and Tyr (35). While MKK4 can activate either the JNK or p38 pathways, MKK7 has been shown to function as a specific activator of JNK (36). Microarray analysis reveals an induction of MKK7, but not MKK4, in T/C-28a2 chondrocytic cells subjected to a shear stress level of 20 dyn/cm², with ratios of 3.40 and 8.33 after 1.5 and 24 hr of stimulation, respectively. This pattern of MKK7 gene regulation parallels the expression of the JNK2 as evidenced by clustering algorithms (supplemental figures 1a, 1b), thereby suggesting that MKK7 may be upstream of JNK2 activation (Fig. 6).

Noninvasive, pharmaceutical-based therapies for the treatment of arthritic diseases involve the use of NSAIDs which elicit their effects by inhibiting COX activity and blocking the downstream production of prostanoids, including PGE₂. Although some in vitro studies have suggested possible anabolic effects associated with low concentrations of PGE₂ (37, 38), several lines of evidence indicate that PGE₂ is involved in cartilage erosion and inflammation associated with rheumatoid arthritis (17). Moreover, PGE₂ production in osteoarthritic cartilage has been found to be significantly elevated as compared to that in normal tissue (39). It has been suggested that PGE₂ derived from COX-2 modulates the degradation of the cartilage proteoglycans in human osteoarthritic tissue stimulated with the pro-inflammatory cytokine IL-
1β (40). While it is known that PGE₂ exerts its biological effects via its four prostaglandin receptors, very little is known about their contribution to the pathogenesis of arthritis. It was recently shown that mice lacking the EP4 receptor displayed a resistance to the development of experimentally-induced arthritis (17). In our studies, we provide clear evidence that shear-induced COX-2 activity, but not COX-1, stimulates the synthesis of prostaglandin receptor subtypes EP2 and EP3a1, while no changes were observed for EP1 and EP4 as evidenced by microarray analysis (data not shown). The potential significance of EP2 and EP3a1 receptor subtypes in other models of arthritis (17) deserves further exploration.

In conclusion, using cDNA microarrays coupled with clustering algorithms followed by directed analysis of our candidate pathway, we elucidated a signaling mechanism regulating COX-2 expression in T/C-28a2 chondrocytic cells stimulated with fluid shear as well as downstream targets of COX-2 activity (Fig. 6). To our knowledge, this is the first application of cDNA microarray technology in conjunction with bioinformatics tools to generate a specific hypothesis about a candidate biochemical pathway in mammalian cells followed by its systematic analysis. This approach can further be exploited to propose novel regulatory networks in biological systems.

ACKNOWLEDGMENTS

The authors thank Dr. Mary Goldring (Harvard Medical School) for providing us with the T/C-28a2 cell line, and Dr. Aikaterini Kontrogianni-Konstantopoulos (University of Maryland at Baltimore) and Dr. Shu-Ching Huang (Harvard Medical School) for insightful discussions. This work was support a DuPont Young Professor Award (KK).
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FIGURE LEGENDS

Figure 1: Validation of the Microarray Technique with a Ribonuclease Protection Assay (RPA). Comparison of cDNA microarray intensity ratios (shear/static) and RPA densitometry ratios (normalized to GAPDH) for seven genes, assessed at the 1.5-hr (A) and 24-hr (B) time-points. Values are mean±S.E.M. from three independent experiments. RPA gels show representative bands on each gene for static (control) and shear stress (20 dyn/cm²) conditions.

Figure 2: Effects of shear exposure time on the mRNA expression of JNK2, c-jun, and COX-2 (A) as well as prostaglandin EP3α1 and EP2 receptors (B). TC28a2 cells were sheared at 20 dyn/cm² for the indicated periods of time ranging from 0.75 to 24 hr. In static (control) experiments, TC28a2 cells were exposed to 0 dyn/cm² for 0.75 to 24 hr. Values are mean±S.E.M. for RPA densitometry ratios (shear/static) from three independent experiments.

Figure 3: (A) Effects of shear exposure time on COX-1 and COX-2 protein levels in TC28a2 chondrocytic cells. Cells were exposed to shear stress (20 dyn/cm²) for durations ranging from 0.75 to 24 hr. The constitutively expressed “housekeeping” protein β-actin was probed as a loading control.

COX-2 localization as assessed by immunofluorescence in TC28a2 chondrocytic cells in the absence (B) or presence (C) of shear stress (20 dyn/cm², 24 hr).

Figure 4: Effects of JNK2 transcript inhibition on COX-1, COX-2, JNK2, phosphorylated c-jun, and Total c-jun Protein Levels in T/C-28a2 Chondrocytic Cells. Cells were subjected to shear stress level of 20 dyn/cm² for 24 hr either in the absence or presence of a JNK2
antisense oligonucleotide. As a control, a JNK2 sense oligonucleotide was utilized. In static experiments, T/C28a2 cells were exposed to 0 dyn/cm² for 24 hr. Panel (A) shows a representative western hybridization experiment for COX-1 and COX-2 while panel (B) shows a similar experiment for JNK2, phosphorylated c-jun (P-c-jun), and total c-jun. β-actin was probed as a loading control.

**Figure 5: (A) Effect of Antisense Oligonucleotide and Chemical Inhibition on Gene Regulation.** Panel (A) shows the effects of c-fos, c-jun, and JNK2 antisense oligonucleotide and NS-398 (a specific COX-2 inhibitor) on shear-induced mRNA expression of c-fos, JNK2, c-jun, COX-2, COX-1 and prostaglandin receptors EP3a1 and EP2. Representative RPA gels show bands on each gene for static (control) and shear stress (20 dyn/cm², 24 hr) conditions in the absence or presence of c-fos, c-jun, or JNK2 antisense oligonucleotide (400nM) or NS-398 (30 µM).

**(B) Shear/Static Densitometry Ratios.** Panel (B) shows RPA densitometry ratios (normalized to GAPDH which ranged from 0.97±0.01 to 1.01±0.01 for all treatments) for seven genes, assessed after exposure of T/C28a2 chondrocytic cells to shear for 24 hours in the absence or presence of antisense oligonucleotides or NS398. Values are mean±S.E.M. from three independent experiments.

**(C) Effect of Shear Stress Magnitude on Gene Regulation.** Panel (C) displays the effects of shear stress magnitude on mRNA expression of c-fos, JNK2, c-jun, COX-2, and prostaglandin receptors EP3a1 and EP2 for 24 hr. Static controls were exposed to a magnitude of 0 dyn/cm², and paralleled the shear duration.
Figure 6: Proposed Cascade of Signaling Events that Results from Stimulation of T/C28a2 Chondrocytic Cells with Fluid Shear. Upon activation by high levels of shear stress, JNK2, possibly induced by MKK7, phosphorylates c-jun, which in turn triggers the transcription of COX-2. Products of COX-2 activity such as PGE\textsubscript{2} ultimately upregulate the synthesis of PG receptor EP3a1 and E2 mRNA.
### FIGURE 1

#### (A)

| Gene      | Microarray | RPA      |
|-----------|------------|----------|
| c-fos     | 0.98 ± 0.04 | 1.03 ± 0.08 |
| JNK2      | 3.40 ± 0.22 | 1.83 ± 0.18 |
| c-jun     | 3.18 ± 0.44 | 2.07 ± 0.19 |
| COX-2     | 2.47 ± 0.12 | 1.85 ± 0.14 |
| PG EP3a1  | 1.42 ± 0.04 | 1.42 ± 0.13 |
| PG EP2    | 1.09 ± 0.08 | 0.94 ± 0.08 |
| GAPDH     | 0.98 ± 0.02 | 1.01 ± 0.01 |

#### (B)

| Gene      | Microarray | RPA      |
|-----------|------------|----------|
| c-fos     | 3.53 ± 0.27 | 2.80 ± 0.57 |
| JNK2      | 8.33 ± 0.73 | 7.09 ± 0.39 |
| c-jun     | 3.50 ± 0.53 | 3.58 ± 0.08 |
| COX-2     | 3.95 ± 0.25 | 3.32 ± 0.12 |
| PG EP3a1  | 3.35 ± 0.26 | 3.44 ± 0.31 |
| PG EP2    | 3.44 ± 0.22 | 3.48 ± 0.31 |
| GAPDH     | 0.99 ± 0.02 | 0.97 ± 0.01 |
FIGURE 2
FIGURE 3

A

Shear - + - + - + - + - +
1.5 6 12 16 24 hours

COX-2
COX-1
β-actin

B

C

Static (no flow) – 24 hours
Shear 20 dyn/cm² – 24 hours
FIGURE 4
Figure 5A, B
FIGURE 5C
FIGURE 6
SUPPLEMENTAL FIGURE 1A

Hierarchical clustering analysis output using TIGR Multi-Experiment Viewer (TMEV). Hierarchical clustering (Average Linkage, Euclidean distance metric) was employed to generate the tree diagram. Horizontal row represent individual genes, while vertical columns represent individual experiments. Data analysis reveals that MKK7, JNK2, c-jun, and COX-2 are contained within the same sub-tree structure, while the prostaglandin receptors EP2 and EP3a1 are co-regulated, though residing in a separate sub-tree structure.
Clustering analysis results obtained using Self-Organizing Maps (SOMs) (TMEV). The gene expression data were clustered using Self-Organizing Maps (SOMs, Euclidean distance metric, 3x3 Hexagonal Topology) to verify the results obtained using pure hierarchical clustering. MAPKK7, JNK2, c-jun, COX-2, and both PG receptors EP2 and EP3a1 were contained within the same cluster, thus supporting the hypothesis that these genes are likely co-regulated. Furthermore, hierarchical analysis within the aforementioned cluster suggested an identical regulatory pattern when compared to pure hierarchical clustering, thus indicating that the regulatory network is conserved independent of the algorithm implemented. The replication of the clustering results therefore prompted the construction of the COX-2 regulatory network.

SUPPLEMENTAL FIGURE 1B
