Regulation of hyaluronan synthase-2 expression in human intestinal mesenchymal cells: mechanisms of interleukin-1β-mediated induction

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Ducale, Ashley E., Susan I. Ward, Tracey Dechert, and Dorne R. Yager. Regulation of hyaluronan synthase-2 expression in human intestinal mesenchymal cells: mechanisms of interleukin-1β-mediated induction. Am J Physiol Gastrointest Liver Physiol 289: G462–G470, 2005. First published January 27, 2005; doi:10.1152/ajpgi.00494.2004.—Elevated levels of hyaluronan are associated with numerous inflammatory diseases including inflammatory bowel disease. The purpose of this study was to determine whether a cause and effect relationship might exist among proinflammatory cytokines, IL-1β, TNF-α, IFN-γ, or transforming growth factor-β (TGF-β) and hyaluronan expression in human JDMC and, if so, to identify possible mechanisms involved in the induction of hyaluronan expression. TGF-β, TNF-α, and IFN-γ had little or no effect on hyaluronan production by these cells. Treatment with IL-1β induced an approximate 30-fold increase in the levels of hyaluronan in the medium of human jejunal-derived mesenchymal cells. Ribonuclease protection analysis revealed that steady-state transcript levels for hyaluronan synthase (HAS)2 were present at very low levels in untreated cells but increased as much as 18-fold in the presence of IL-1β. HAS3 transcript levels were also increased slightly by exposure of these cells to IL-1β. Expression of HAS1 transcripts was not detected under any condition in these cells. IL-1β induction of hyaluronan expression was inhibited in cells transfected with short interfering RNA corresponding to HAS2 transcripts. Inhibitors of the p38 and ERK1/2 mitogen-activated pathways but not JNK/SAPK blocked the IL-1β-mediated induction of hyaluronan expression and the increase in HAS2 transcript expression. These results suggest that IL-1β induction of HAS2 expression involves multiple signaling pathways that act in concert, thus leading to an increase in expression of hyaluronan by jejunal-derived mesenchymal cells.

mitogen-activated pathways; inflammation; jejenum; nucleofection

HYALURONAN IS A UBQUITOUS polysaccharide composed of a repeat of the alternating disaccharide β-D-glucuronic acid(1→3)-β-d-N-acetylglosaminic acid(1→4). The linear polymers of this glycosaminoglycan can contain tens of thousands of sugar residues (up to ~10^6 Da). Hyaluronan is a major constituent of the extracellular matrix and is particularly abundant in the dermis, synovial fluid, and vitreous body(23). It is the major glycosaminoglycan of the gut with levels of ~600 ng/mg dry weight of the small intestine (5, 56). Ubiquitously expressed, hyaluronan has been localized in extracellular, cell surface, and intracellular environments(28).

Until recently, hyaluronan was believed to primarily play an important but passive structural role in the maintenance of tissue architecture (23). It probably exists as a stiff random coil that occupies a large hydrodynamic domain and preferentially excludes large macromolecules (23). Over the years, much work has focused on the interaction of hyaluronan with various extracellular matrix molecules such as aggrecan and versican (23). These interactions have proven critical for tissue volume changes and the creation and stability of cell-free spaces (36, 41, 42, 55, 59, 62). Another area of interest is its interaction with a number of cell surface receptors, most notably CD44 (18). Binding of hyaluronan to CD44 provides a link to the cytoskeleton (via ankyrin) and stimulates intracellular signaling via protein tyrosine kinase, Rac, Ras, protein kinase C, and phosphoinositide 3′-kinase (22, 33, 43). Another receptor, receptor for hyaluronan-mediated motility (RHAMM), distributes to the cell surface and to intracellular compartments (6, 12). Cell-surface RHAMM-hyaluronan binding triggers activation of protein tyrosine kinases, ERK kinases, and protein kinase C (27, 37).

Hyaluronan has been shown to induce several fundamental cell processes. In macrophages and dendritic cells, intermediate-molecular weight hyaluronan can induce nitric oxide synthase (39). Furthermore, hyaluronan can induce dendritic cell apoptosis (19, 20, 35, 38). Oligomeric hyaluronan (8–16 disaccharides) induces angiogenesis in a chick cornea assay (58). Hyaluronan fragments can induce the expression of monocyte chemokines (40). Hyaluronan and T cell-CD44 interactions have also been shown to play a role in leukocyte extravasation (17).

Elevated hyaluronan levels are associated with virtually all disease processes involving inflammation. This includes rheumatoid arthritis, periodontitis, scleroderma, psoriasis, and inflammatory bowel disease (2, 19, 20, 38, 61). Various proinflammatory cytokines have been shown to influence hyaluronan expression in cells originating from connective tissue (29, 34, 53). IFN-γ, IL-1, and transforming growth factor-β have been shown to stimulate hyaluronan production of orbital fibroblasts (60). IL-1α, IL-1β, and TNF-α also stimulate the expression of hyaluronan by synovial fibroblasts (8). We and others have shown that IL-β and TNF-α stimulate the expression of hyaluronan in human dermal fibroblasts (34, 45, 46).

Hyaluronan is synthesized at the inner face of the plasma membrane by one of three distinct hyaluronan synthases (HAS) (31, 44). HAS1, HAS2, and HAS3 are encoded on separate chromosomes but possess similar amino acid and structural similarities (50, 51). There is evidence that these enzymes have distinct functions. Recombinant HAS enzymes display differences in stability and Km values for the substrates UDP-N-acetylglucosamine and UDP-glucuronic acid(31). The recombinant HAS enzymes also synthesize hyaluronan of different molecular weights, with HAS3 products being smaller than those of both HAS1 and HAS2 (31). HAS expression has been
shown to be cell-type and context specific (32). For example, HAS2 transcripts are expressed constitutively in human dermal-derived fibroblasts whereas, HAS1 and HAS3 transcripts are found in human keratinocytes (34, 49). Human fibroblast-like synoviocytes were shown to express HAS2 and HAS3 transcripts, but HAS1 levels were virtually undetectable (52).

The association of elevated hyaluronan levels in inflammation and the ability of hyaluronan to interact with and activate various leukocytes suggest that hyaluronan may have an important role in the pathogenesis of chronic inflammatory conditions such as atherosclerosis, chronic wounds, Crohn’s disease, and inflammatory bowel disease. Individuals with inflammatory bowel disease express increased luminal hyaluronan (2, 11). Hyaluronan staining is greatly pronounced in the affected areas of ulcerative colitis and Crohn’s disease compared with adjacent normal tissues (16). There is also evidence that intestinal mesenchymal cell-leukocyte interactions that occur in inflammatory bowel disease are hyaluronan dependent (13–15). This suggests a link between intestinal inflammation and hyaluronan expression. The purpose of this study was to determine the effects of proinflammatory cytokines on the expression of hyaluronan and the HASs in jejunal-derived mesenchymal cells and to begin characterizing the mechanisms used by these mediators to regulate hyaluronan expression. Very few studies to date have examined intracellular signaling pathways involved in the regulation of hyaluronan synthase expression and hyaluronan synthesis (52). Given previous reports of HAS regulation by MAPK pathways and utilization of these pathways by IL-1β, we examined the role of MAPK pathways in IL-1-β-mediated regulation of hyaluronan and HAS expression in jejunal-derived mesenchymal cells.

MATERIALS AND METHODS

Materials. Recombinant human IL-1β, TNF-α, IFN-γ, and transforming growth factor (TGF)-β were purchased from R&D Systems (Minneapolis, MN). The p38 inhibitor SB 202190 (IC50 = 350 nM), mitogen-activated/extracellular response kinase-1 (MEK1) inhibitor PD 98059 (IC50 = 2 μM), JNK inhibitor II SB 600125 (IC50 = 90 nM for JNK-3), and biotinylated hyaluronan binding protein (HAbp) were purchased from Calbiochem (San Diego, CA). HAS1 and HAS2 siRNAs were obtained from Calbiochem (San Diego, CA). HAS1 and HAS2 siRNAs were obtained from Calbiochem (San Diego, CA). 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS) were obtained from Pierce Endogen (Rockford, IL). Umbilical cord hyaluronan, anti-biotin horseradish peroxidase, and 3′,5′-tetramethylbenzidine (TMB) were obtained from Sigma (St. Louis, MO).

Cell culture. Human mesenchymal cells were established from the mucosalis externa of jejunal using previously established procedures (3, 26). The jejunal were obtained from consenting subjects undergoing gastric bypass surgery. This study was approved by the Virginia Commonwealth University Office of Research Subjects Protection. These cells were positive for α-smooth muscle actin, desmin, and vimentin expression. A total of three independently established jejunal-derived mesenchymal lines were used in this study. Culture media, supplements, and subculturing reagents were purchased from Invitrogen (Carlsbad, CA). Cells were cultured in DMEM (4,500 mg/ml glucose + pyruvate) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C in a humidified incubator containing 5% CO2. For experiments, cells between passages 5 and 10 were used at confluence. Control and test cell monolayers were fed with fresh medium supplemented with 1× ITS+ (Invitrogen) in place of serum 24 h before stimulation. Concentrations of IL-1β (120 pM), TNF-α (1.2 nM), TGF-β1 (200 pM), and IFN-γ (1.8 nM) had been previously shown to be optimal for hyaluronan stimulation (34). Stimulation with the various mediators was carried out for 24 h at 37°C and 5% CO2 unless otherwise indicated. For the MAPK inhibitor studies, each inhibitor was used at a concentration threefold higher than its IC50. Inhibitors or vehicle only (dimethylsulfoxide) were added to the 1× ITS+ culture medium 1 h before IL-1β stimulation.

Hyaluronan assay. Hyaluronan levels were quantified using one of two procedures. The first method involved a slot-blotting approach. Known amounts of hyaluronan (3–30,000 ng/ml) were applied to the slots to generate a standard curve. For the samples, 15 μl of culture media diluted in 85 μl of 0.05% Tween-PBS were applied to a nitrocellulose membrane that had been previously derivatized with 1% cetylpyridinium chloride in 30% isopropanol for 5 min and washed with 150 mM NaCl for 15 min. After application of the standards and samples, the membrane incubated at 37°C for 6 h in blocking solution (100 mM sodium acetate, pH 6.0, 1 M NaCl, 0.05% Tween 20, 2% nonfat dry milk, and 0.1% sodium azide) after which 0.5 μg/ml biotinylated HAbp diluted 1:200 in blocking solution was added to the membrane and allowed to incubate at 4°C overnight. Antibiotin diluted 1:2,000 in blocking solution with sodium azide was added for 1 h after washing the membrane five times for 5 min each. Unbound material was removed by washing five times with blocking solution without sodium azide before visualization. Peroxidase activity was visualized by chemiluminescent detection using the Western Lightning detection kit (Perkin Elmer, Boston, MA) and subsequent exposure to Kodak X-omat film.

The second method of hyaluronan quantification involved a competitive enzyme-linked immunosorbent-like assay modified from Frost and Stern (24). Carboxyls of 2 mg of umbilical cord hyaluronan were modified to amine reactive NHS esters by incubating in 20 μl with 0.123 mg/ml EDC and 0.184 mg/ml sulfo-NHS. The modified hyaluronan (100 μg/well) was immediately added to Covalink-NH microtiter plates (NUNC, Placerville, NJ). The hyaluronan was allowed to bind for 2 h at room temperature and then removed by washing four times with PBS containing 0.05% Tween 20. Aliquots of samples brought to 100 μl with PBS-Tween 20 were added in quadruplicate wells. Known amounts of hyaluronan (3–30,000 ng/ml) were also used to generate a standard curve. One hundred microliters of 0.5 μg/ml biotinylated HAbp were added and mixed to each well. Binding was permitted to occur overnight at room temperature. Unbound material was removed by washing four times with PBS-Tween 20. One hundred microliters of anti-biotin horseradish peroxidase diluted 1:2,000 with PBS-Tween 20 containing 1% BSA were added and incubated at room temperature for 1 h. Unbound material was removed by washing four times with PBS-Tween 20. Levels of remaining horseradish peroxidase were determined by adding 100 μl of TMB. The reaction was stopped by adding 100 μl 1 M H2SO4 and the absorbance at 450 nm.

Analysis of hyaluronan molecular weight. The size distribution of hyaluronan produced by jejunal-derived mesenchymal cells (JDMC) was measured using a method described by Armstrong and Bell (5). Aliquots of medium were applied to 0.5% agarose gels in buffer containing 40 mM Tris, 5 mM sodium acetate, and 0.8 mM sodium EDTA, pH 7.9. Hyaluronan of known molecular weights was used as standard. Samples were subjected to electrophoresis at 50 V until the bromophenol blue dye front had migrated 15 cm. After electrophoresis, Immobilon-NC (Millipore, Bedford, MA) was treated with 1% cetylpyridinium chloride dissolved in 30% isopropanol for 5 min. The filter was then incubated in 50 ml 150 mM NaCl for 15 min on a shaker. Hyaluronan was transferred overnight from the gel to the filter by capillary wicking using 100 mM Tris-acetate (pH 7.3). After transfer, the filter was blocked for 6 h at 37°C in 100 mM sodium acetate, pH 6.0, 1 M NaCl, 0.05% Tween 20, 2% nonfat dry milk, and 0.1% sodium azide. The filter was then transferred into fresh blocking buffer containing biotinylated-hyaluronan binding protein diluted 1:200 and incubated overnight at 4°C. Filters were washed five times.
with the blocking buffer (without azide) for 5 min each to remove unbound biotinylated-hyaluronan binding protein. Anti-biotin antibody conjugated with horseradish peroxidase diluted 1:10,000 in blocking buffer without azide was added to the filter and incubated at 23°C for 1 h. The filter was washed five times in blocking buffer without azide and peroxidase activity visualized by chemiluminescent detection using the Western Lightning detection kit and exposing to Kodak X-OMAT film.

Analysis of mRNA levels. Total RNA was isolated from monolayer cultures using single-step acid guanidinium thiocyanate organic extraction (10). Alcohol-precipitated RNA was further purified by washing once with 2 M LiCl. To generate templates for ribonuclease protection assays, RT-PCR was performed using human HAS1-, HAS2-, and HAS3-specific primers (HAS1 NM_001523.1 nct 893–1094; HAS2 NM_005328.1 nct 535–929; HAS3 AF234839 nct 861–1252). PCR products were inserted into the Bluescript pSK vector (Stratagene, La Jolla, CA) and sequenced in their entirety to confirm their identity. Relative transcript levels were determined by a ribonuclease protection assay using the MAXIscript kit and RPA III kit (Ambion). Protected products were resolved by electrophoresis on 6% polyacrylamide gels containing 8 M urea in buffer containing 89 mM Tris-borate, pH 8.3, and 2 mM EDTA. Gene-specific mRNA levels were detected and quantified using a Bio-Rad molecular imager FX and Quantity One quantitation software (Bio-Rad Laboratories, Hercules, CA). Variations in signal strength resulting from differences in the amount of total RNA loaded in each well were corrected for normalization to cyclophilin mRNA levels.

Knockdown analysis of HAS2. Chemical-synthesized sense and antisense RNAs corresponding to the HAS1 and HAS2 cDNA sequences (041904si: GGG-AGG-GUA-UUU-GGU-Ctt, nucleotides 1957–1975 from NM_001523.1 NCBI; and 041908si: GGU-UGG-GUA-UUU-AUU-GGU-Ctt, nucleotides 1162–1180 from NM_005328.1 NCBI) were purchased from Ambion. For siRNA transfection, cells at exponential growth were harvested by trypsinization and resuspended at 5.0 × 10^5 cells/100 μl in Nucleofector solution V (Amaxa, Gaithersburg, MD). Cells were mixed with 1.0 μl of 100 μM siRNA and 3.0 μl of 1 μg/μl pEGFP-C1 [BD Biosciences Clontech (San Diego, CA)]. Nucleofection was performed in a 0.4-ml cuvette and Nucleofector (Amaxa) using the manufacturer’s instructions. After being pulsed, cells were mixed with 500 μl of DMEM containing 10% FBS and dispensed into triplicate 30-mm culture dishes containing an additional 2.0 ml of medium. After cells adhered overnight at 37°C and 5% CO_2, they were collected by trypsinization, and those expressing the EGFP-C1 protein isolated via fluorescence-associated cell sorter (FACS) analysis. Cells expressing EGFP-C1 were replated and incubated overnight, after which they were stimulated with 120 pM IL-1β. After IL-1β treatment for 24 h, culture media was collected and hyaluronan content of the media was quantified using the slot-blotting approach described in Hyaluronan assay.

Flow cytometry. Twenty-four hours after nucleofection, JDMCs were trypsinized, washed in PBS, and cells expressing EGFP were isolated by sterile sorting using the Coulter Epics Elite ESP flow cytometer with a standard filter set. With all the siRNA:pEGFP cotransfections performed, FACS analysis demonstrated transfection efficiencies in the 45–58% range.

Data analysis. Hyaluronan levels produced under different conditions were analyzed by ANOVA followed by a post hoc test. A probability of P < 0.05 was considered to be statistically significant. Data are presented as means (SD). A statistical computer software program was used for analyses (SigmaStat, Systat, Richmond, CA).

RESULTS

Effects of proinflammatory mediators on hyaluronan accumulation. Elevated hyaluronan levels are associated with inflammatory processes including inflammatory bowel disease (1). Therefore, the ability of several proinflammatory mediators to modulate hyaluronan expression in jejunum-derived mesenchymal cells was examined. Monolayer cultures were made quiescent by washing and replacing serumized medium with medium supplemented with 1X ITS+. After an overnight incubation, the medium was removed and replaced with ITS+ media containing 120 pM IL-1β, 1.2 nM TNF-α, 200 pM TGF-β1, or 1.8 nM IFN-γ. After incubation for 24 h, a competitive enzyme-linked immunosorbent-like assay was used to compare hyaluronan levels in the culture medium of unstimulated cells with those of cells treated with the various proinflammatory mediators. Unstimulated jejunum-derived mesenchymal cells produce little hyaluronan in vitro (Fig. 1, lane 1). Treatment with TNF-α, TGF-β, or IFN-γ did not significantly increase hyaluronan levels in the medium (Fig. 1, lanes 3–5). In contrast, treatment with IL-1β or IL-1β plus TNF-α induced a large increase in hyaluronan levels in the medium (~30- and 100-fold, respectively; P ≤ 0.05; Fig. 1, lanes 2 and 6). Less than 10% of the total hyaluronan was determined to be cell associated and did not vary with treatment.

Effects of proinflammatory mediators on HAS transcript levels. Experiments were performed to identify the HAS isoform involved in the IL-1β-mediated upregulation of hyaluronan in jejunum-derived mesenchymal cells. We have previously observed in dermal-derived fibroblasts that IL-1β-induced hyaluronan production was accompanied by an increase in HAS1 transcript levels (34). In unstimulated cells, ribonuclease protection assays did not detect HAS1 or HAS3 transcripts and only very low levels of HAS2 transcripts. Cells stimulated with 24 h with IL-1β expressed no detectable HAS1 transcripts (data not shown), low levels of HAS3 transcripts, but markedly elevated levels of HAS2 transcripts (Fig. 2, A and B). Closer examination of HAS2 transcript levels in cells stimulated with IL-1β, TNF-α, TGF-β, and IFN-γ indicated that the effect was specific to IL-1β (18-fold), although a
combination of IL-1β and TNF-α induced HAS2 transcript levels (27-fold) in a manner that mirrored their synergistic effect on hyaluronan expression (Fig. 2, A and B).

We have previously noted that IL-1β induction of HAS1 transcript levels in dermal fibroblasts is transient, peaking at ~8–10 h after the addition of IL-1β and returning to nearly baseline levels by 24 h. Therefore, the time course of IL-1β induction of HAS2 transcript levels was also examined. In contrast to HAS1 in dermal fibroblasts, HAS2 transcript levels did not begin to rise until after 8 h of exposure to IL-1β but then continued to increase thereafter (2.5-fold at 12 h, 3-fold at 24 h, and 6-fold at 48 h; Fig. 3).

**HAS2 knockdown blocks the IL-1β induction of hyaluronan accumulation.** The IL-1β induction of HAS2 transcript levels correlated well with the IL-1β induction of hyaluronan expression by jejunum-derived mesenchymal cells. To demonstrate that HAS2 was indeed the HAS isoform primarily involved in the IL-1β induction of hyaluronan expression, jejunum-derived mesenchymal cells were cotransfected with pEGFP-C1 and HAS2-specific siRNA. After 20 h, flow cytometry was used to select for cells expressing EGFP, the cells were replated and treated with 120 pM IL-1β for 24 h. The HAS2-specific siRNA inhibited the IL-1β induction of hyaluronan expression by 35–45% compared with IL-1β-treated control transfectants. As a negative control, cells were also transfected with HAS1-specific siRNA (Fig. 4). This finding provides evidence for a direct link between IL-1β induction of HAS2 gene expression and the observed increase in hyaluronan production by these cells.

**Utilization of MAPK inhibitors to identify IL-1β-induced pathways involved in regulating hyaluronan and HAS2 transcript levels.** Use of MAPK pathways have been implicated in a variety of IL-1β models (9, 57). At least one MAPK pathway...
has been shown to be involved in the TGF-β regulation of hyaluronan expression in fibroblast-like synoviocytes (52). Therefore, a pharmacological approach was used to investigate the involvement of one or more of the three major MAPK pathways in the IL-1β induction of hyaluronan and HAS2 transcripts in jejunum-derived mesenchymal cells. Cells were pretreated for 1 h with 6.0 μM PD98059, 1.0 μM SB202190, and 300 nM SP600125 to inhibit the p42/44, p38 MAPK, and JNK (1, 2, and 3) pathways, respectively. IL-1β (120 pM) was added, and cells were allowed to incubate overnight at 37°C. Hyaluronan levels that accumulated in the 24-h time period were determined as well as HAS2 transcript levels. Inhibition of the p42/44 pathway reduced hyaluronan expression to ~37% of that observed in cells treated with IL-1β only (Fig. 5). Inhibition of the p38 MAPK pathway reduced hyaluronan expression to only ~7% of what was produced by cells treated with IL-1β only (Fig. 5). There was no significant reduction in hyaluronan expression (92% of IL-1β alone) when the JNK pathway was inhibited. Hyaluronan levels expressed by cells treated with all three inhibitors were not significantly different than those treated only with the inhibitor of the p38 pathway.

Likewise, the p42/44 and p38 MAPK inhibitors blocked the IL-1β induction of HAS2 transcripts, whereas the JNK inhibitor had little effect (Fig. 6, A and B). None of the MAPK inhibitors appeared to significantly alter cyclophilin transcript levels.

Molecular weight profile of IL-1β-mediated induced hyaluronan in jejunum-derived mesenchymal cells. There is evidence that the molecular weight of nascent hyaluronan is dependent on the HAS isoform involved in its synthesis (31). The molecular weight of hyaluronan has been shown to influence its biological function (21, 30, 40–42, 52, 55, 59, 62). The effects of IL-1β and TNF-α on the molecular mass of hyaluronan present in the culture media was examined. Media fractions were size fractionated by electrophoresis in 0.5% agarose gels. The hyaluronan was transferred to a derivatized nitrocellulose membrane and then probed using a biotinylated hyaluronan-binding protein. As expected, little or no hyaluronan was detected in media from untreated cells (Fig. 7, lane 4). The medium from cells treated with IL-1β or IL-1β plus TNF-α contained hyaluronan with an apparent molecular weight greater than 1.106 Da (Fig. 7, lanes 5 and 6). In addition, medium from cells treated with both IL-1β and TNF-α also generated a secondary peak of hyaluronan centered around 5.105 Da. Pretreatment with PD98058, SB202190, or the combination of all three MAPK inhibitors before adding IL-1β decreased the entire molecular weight range of hyaluronan (Fig. 7, lanes 7, 8, and 10).
MECHANISMS OF HYALURONAN REGULATION

Fig. 7. Molecular weight profile of hyaluronan produced by JDMCs treated with proinflammatory cytokines and growth factors. The hyaluronan from culture media collected after treatment with the mediators for 24 h was fractionated by 0.5% agarose gel electrophoresis. Hyaluronan was detected using the enhanced chemiluminescence detection system with biotinylated hyaluronan-binding protein and anti-biotin antibody conjugated with horseradish peroxidase. Standards of molecular weights 8.5 × 10^5 (lane 1), 3.5 × 10^5 (lane 2), and 1.5 × 10^5 Da (lane 3) were used for comparison. Samples included untreated cells (lane 4), 120 pM IL-1β (lane 5), 120 pM IL-1β, and 1.2 nM TNF-α (lane 6), 120 pM IL-1β 1 h posttreatment with PD98059 (lane 7), SB202190 (lane 8), JNK II (lane 9), and PD98059, SB202190, and JNK II (lane 10).

DISCUSSION

A persistent inflammatory component is a hallmark of many diseases such as cancer, diabetes, osteoporosis, rheumatoid arthritis, inflammatory bowel disease, and ulcerative colitis. Studies using an induced colitis rabbit model demonstrated early IL-1 synthesis with levels correlating to the degree of tissue inflammation and destruction (4). Given this, it is important to elucidate the downstream effects of persistent IL-1 and the pathways used. Once identified, these mechanisms could be potential therapeutic targets when considering treatment for these pathologies. Hyaluronan has been almost universally linked to processes involving an inflammatory component such as wound healing, rheumatoid arthritis, and cancer progression (41, 55). Given that proinflammatory cytokines have been demonstrated to modulate hyaluronan levels in other cell types, the present experiments sought to determine whether there is a link between increased hyaluronan and exposure of jejunum-derived mesenchymal cells to several proinflammatory mediators. IL-1β and TNF-α play a central role during the inflammatory phase of pathological processes and have been shown to exert a similar broad range of physiological effects (47).

Three HASs, the enzymes responsible for hyaluronan synthesis, have been identified, but the regulatory mechanisms governing their expression and activities have only begun to be elucidated. Under normal culture conditions, jejunum-derived mesenchymal cells produce low levels of hyaluronan, and this is largely reflected by the observed transcript levels for HAS1, HAS2, and HAS3. In this study, neither TGF-β, IFN-γ, nor TNF-α stimulated hyaluronan expression or induced a significant increase in HAS2 transcripts. In contrast, IL-1β induced a significant increase in HAS2 transcript levels and hyaluronan expression by jejunum-derived mesenchymal cells. Interestingly, although TNF-α by itself had little or no discernible effect on hyaluronan expression, inclusion of this cytokine with IL-β appears to have a synergistic effect. Both of these cytokines would normally be expected to be present in most processes that involve inflammation.

Induction of HAS1 transcripts by IL-1β in dermal fibroblasts is transient, with a peak at ~8 h after which the transcript levels approach baseline levels by 24 h. The time course of IL-1β induction of HAS2 transcript levels was not as immediate, and this response is sustained. This provides an indication that the response of HAS genes to IL-1β is cell-type specific and that the mechanisms involved may differ. HAS3 transcript levels are increased in response to IFN-γ and decreased with TGF-β in cultured keratinocytes, whereas HAS1 and 2 levels were unchanged (49). In human dermal fibroblasts, basal transcript levels of HAS2 were significantly suppressed with the administration of glucocorticoids, therapeutic agents with pronounced side effects such as organ atrophy (62). When measuring HAS transcript levels in human fibroblast-like synoviocytes, TGF-β potently activated HAS1 but suppressed HAS3 in a dose-dependent manner (52).

The changes seen in HAS2 transcript levels induced by IL-1β mirrored that of hyaluronan levels, suggesting that HAS2 expression is responsible for IL-1β-mediated upregulation of hyaluronan in jejunum-derived mesenchymal cells. However, hyaluronan levels can be increased in any given system by several mechanisms. Hyaluronidase, the enzyme responsible for hyaluronan degradation, can be downregulated. Receptor-mediated uptake can also potentially influence hyaluronan levels. Alterations in the precursor pool required for hyaluronan synthesis might also be altered by IL-1β. The siRNA knockdown approach suggests that HAS2 expression is directly involved in the increase in hyaluronan synthesis that is induced by IL-1β.

Several IL-1-responsive protein kinase pathways have been identified, including the three major MAPK pathways consisting of p42/44, p38, and JNK. The p42/44 and p38 pathways have been shown to be required for IL-1β-mediated IL-8 gene expression in human retinal pigment epithelial cells (7). IL-1β stimulated p38 phosphorylation in rat islet cells resulting in phosphorylation of activating transcription factor-2 and cAMP-responsive element-binding protein (48). The results in this study indicated that the p38 and p42/44 pathways, but not the JNK pathway, are involved in IL-1β-mediated induction of hyaluronan and HAS2 transcript expression in jejunum-derived mesenchymal cells.
The HAS isozymes have distinct enzymatic properties as well as individual roles during biological processes. Analysis of hyaluronan product size demonstrates chain lengths of up to $2 \times 10^6$ Da can be produced by HAS1 and HAS2, whereas the product generated by HAS3 is significantly smaller, $<2 \times 10^5$ to $3 \times 10^5$ Da. High-molecular weight hyaluronan has been linked to growth inhibition, whereas low-molecular weight hyaluronan has been linked to cell proliferation, initiation of signaling cascades, angiogenesis, and inflammatory responses (54). Given the link between hyaluronan chain length and biological function, this study also examined the effects of IL-1β on hyaluronan molecular weight. The results demonstrated that IL-1β treatment resulted in production of hyaluronan with an apparent molecular weight range of 0.85–1.1 kDa.

Through activation of signal-transduction pathways, IL-1β influences gene expression by modulating the activity of a number of transcription factors. Several of these factors include AP-1, NF-κB, and NF-IL6. NF-κB is a member of the Rel family of transcription factors and is widely implicated in inflammatory processes. It is activated on phosphorylation and subsequent ubiquination of its bound inhibitor IκB. On dissociation from its inhibitor, NF-κB translocates to the nucleus where it binds to its consensus sequence, modulating gene expression. AP-1 is a heterodimeric complex that is largely regulated at the posttranscriptional level via phosphorylation. Both factors are downstream targets of the MAPK pathways and thus can potentially regulate HAS and hyaluronan levels. In a human lung myofibroblast cell line, TNF-α treatment increased HAS activity and hyaluronan levels via activation of the p50/p65 NF-κB complex (42). Interestingly, examination of sequences proximal to the HAS2 transcriptional start site reveals two AP-1 consensus sequences. Given this, it is plausible to suggest that in jejunal-derived mesenchymal cells, IL-1β regulates HAS and hyaluronan levels via transcription factor activation. This hypothesis needs further investigation via DNA-binding and competition experiments.

Recent evidence has suggested a role for MAPK pathways in cytokine-induced mRNA stability. Recent reports (59) have implicated p38 MAPK pathway in IL-1β-induced mRNA stability. Preliminary results from this laboratory suggest a posttranscriptional mechanism is involved in IL-1β-mediated increases in HAS2 transcript levels. HAS2 contains adenosine and uridine-rich elements (AREs), sequences known to confer message instability (25). IL-1β has been shown to influence stability of mRNAs containing AREs. Therefore, it is important to conduct studies looking at the role of IL-1β in HAS2 mRNA stability and the mechanisms used to carry out this function.

In summary, we demonstrated that IL-1β treatment can lead to increased levels of HAS2 transcripts with a resultant increase in hyaluronan levels. Blocking either the ERK or p38 MAPK pathways completely suppresses both HAS2 and hyaluronan increases by IL-1β. In the future, we would like to further elucidate the mechanisms used by IL-1β to increase HAS and hyaluronan levels. Experiments need to examine either possibility that IL-1β uses transcriptional or posttranscriptional mechanisms to affect hyaluronan biology in jejunum-derived mesenchymal cells.

GRANTS

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