Polyinosine-Polycytidylic Acid Stimulates Versican Accumulation in the Extracellular Matrix Promoting Monocyte Adhesion

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Viral infections are known to exacerbate asthma and other lung diseases in which chronic inflammatory processes are implicated, but the mechanism is not well understood. The viral mimetic, polyinosine-polycytidylic acid, causes accumulation of a versican- and hyaluronan-enriched extracellular matrix (ECM) by human lung fibroblasts with increased capacity for monocyte adhesion. The fivefold increase in versican retention in this ECM is due to altered compartmentalization, with decreased degradation of cell layer-associated versican, rather than an increase in total accumulation in the culture. This is consistent with decreased mRNA levels for all of the versican splice variants. Reduced versican degradation is further supported by low levels of the epitope, DPEAAE, a product of versican digestion by a disintegrin-like and metalloproteinase with thrombospondin type 1 motif enzymes, in the ECM. The distribution of hyaluronan is similarly altered with a 3.5-fold increase in the cell layer. Pulse-chase studies of radiolabeled hyaluronan show a 50% reduction in the rate of loss from the cell layer over 24 hours. Formation of monocyte-retaining, hyaluronidase-sensitive ECMs can be blocked by the presence of anti-versican antibodies. In comparison, human lung fibroblasts treated with the cytokines, IL-1 β plus TNF- α , synthesize increased amounts of hyaluronan, but do not retain it or versican in the ECM, which, in turn, does not retain monocytes. These results highlight an important role for versican in the hyaluronan-dependent binding of monocytes to the ECM of lung fibroblasts stimulated with polyinosine-polycytidylic acid.

Keywords: extracellular matrix; versican; a disintegrin-like and metalloproteinase with thrombospondin type 1 motif; monocytes; lung inflammation

The persistent nature of asthma results, in part, from the presence of an extracellular matrix (ECM), which supports the proliferation and migration of resident cells in addition to invasion and retention of leukocytes as part of the inflammatory response in the lung (1, 2). This ECM, enriched in two specific molecules, versican and hyaluronan, is synthesized by various lung cells in response to cytokines, other inflammatory mediators, and infectious agents. Previous studies have shown that hyaluronan and versican accumulate in the airways of humans with pulmonary fibrosis (3), acute respiratory distress syndrome (4), lymphangioleiomyomatosis (5), chronic obstructive pulmonary disease (6), and asthma (7), and in animal

CLINICAL RELEVANCE

The findings in this article indicate that a specific extracellular matrix molecule can bind inflammatory cells, and thus represents a potential target for clinical intervention in diseases of an inflammatory nature.

models of asthma (8). Frequently, these components are found together in regions of inflammatory infiltrates, suggesting a causal relationship between the accumulation of these ECM components and leukocyte accumulation (4, 8, 9). However, the importance of each of these components to each other in serving as attachment sites for leukocytes, and the mechanism(s) responsible for their accumulation in the ECM, are poorly understood.

Viruses have been clearly implicated in the pathogenesis of asthma, and may cause this and other lung diseases. For example, it is well accepted that respiratory syncytial virus (RSV) and rhinovirus are causes of childhood asthma (10). In addition, a number of viruses have also been associated with exacerbations of asthma, including RSV, rhinovirus, influenza, and parainfluenza (11). RSV is a retrovirus that stimulates expression by airway epithelial cells of Toll-like receptor-3, the receptor for double-stranded RNA, which also binds the viral mimetic, polyinosine-polycytidylic acid (poly I:C) (12). However, it is unclear how viral infection causes or worsens lung diseases. One possibility is that viruses interact with the resident cells of the lung to alter their production of ECM in such a way as to create a matrix that interacts with inflammatory cells. Consistent with this, studies by de la Motte and colleagues (13) with intestinal mucosal smooth muscle cells have shown that poly I:C induces formation of a hyaluronan-dependent ECM complex that binds monocytes, suggesting proinflammatory properties. An alternative view is that, in some instances, this ECM may be anti-inflammatory by virtue of its ability to sequester leukocytes, preventing their activation and cytokine secretion (14).

IL-1 β , TNF- α , and IFN are ubiquitous mediators in many inflammatory diseases, and have been implicated in asthma (15). We have previously demonstrated that IL-1 β plus TNF- α will cause a synergistic increase in hyaluronan accumulation in human lung fibroblast (HLF) cultures (16). Although it has not been shown whether versican increases similarly in these IL-1 β plus TNF- α -treated HLF cultures, we have shown that IL-1 β decreases versican production in aortic smooth muscle cells, suggesting that each of these two ECM components are differentially regulated by specific cytokines, and do not always accumulate together (17). Although hyaluronan is generally viewed as a marker of inflammation, its function is complex, and varies with molecular size and possible postsecretion cross-linking with other molecules. Hyaluronan can regulate cell

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proliferation in either a positive or negative fashion, and may, under some circumstances, inhibit inflammatory responses (14, 18).

A number of studies have demonstrated a key role for hyaluronan in the inflammatory response (19, 20), but considerably less is known about versican, a chondroitin sulfate proteoglycan that interacts specifically with hyaluronan (21). In this study, we demonstrate that versican together with hyaluronan dramatically increases in the ECM of cultures of HLFs treated with the viral mimetic, poly I:C, but not in similar cultures treated with IL-1 β plus TNF- α . Colocalization of versican and hyaluronan was prominent in the ECM of poly I:C-treated cultures, whereas no colocalization of these components was observed in IL-1 β plus TNF- α -treated or control cultures. Furthermore, we show that this increase is mainly due to reduced degradation and processing of versican as well as hyaluronan. Confirmation that poly I:C reduced the degradation of versican was the reduction of the DPEAAE fragment of versican generated by a disintegrin-like and metallopeptidase with thrombospondin type 1 motif (ADAMTS) proteolytic degradation (22) when compared with cytokine-treated controls. Furthermore, cytokine treatment of HLF markedly increased the mRNA for ADAMTS4, a major versican degradative enzyme, whereas poly I:C treatment caused only a minor increase above control level. The concept that poly I:C induces altered processing of hyaluronan by HLF is supported by our observation that, when compared with control or cytokine-treated cells, poly I:C reduces the mRNA transcripts for hyaluronidase (HYAL) 1 and HYAL 2, two of the principle enzymes responsible for hyaluronan degradation (23). In addition, poly I:C reduced the mRNA for CD44, which is the major cell surface receptor for hyaluronan uptake and degradation by cells (24). Furthermore, ECMs that formed in the presence of poly I:C, and that contained both versican and hyaluronan, bound monocytes in a HYAL-sensitive manner, whereas ECMs that contained hyaluronan, but no versican, did not. In fact, extracellular versican was conspicuously absent from IL-1 β plus TNF- α -treated cultures. Antibodies to versican, as well as fragments of hyaluronan, blocked the production of a monocyte-retaining ECM. Furthermore, the steroid, fluticasone, which inhibits hyaluronan synthesis (16), also interfered with the formation of this ECM. These findings indicate that reduced degradation and turnover of both versican and hyaluronan allow the formation of an extensive ECM that supports monocyte adhesion, and that both of these components must be present for monocyte adhesion to occur. This may be one reason that viral infections are capable of causing and exacerbating the inflammatory reaction associated with asthma.

MATERIALS AND METHODS

Cell Culture

HLFs were derived from explants of the human lung after removal of both the pleura and parenchyma. These cells were a generous gift from Professor Ganesh Raghu, Division of Pulmonary and Critical Care Medicine, University of Washington (Seattle, WA). HLFs were isolated, as described previously, in accordance with approval from the human subjects review committee of the institution (25). A second line of HLF was obtained from the American Type Culture Collection (Manassas, VA). HLFs were maintained in Dulbecco's modified Eagle medium (DMEM) high-glucose medium supplemented with 10% FBS (HyClone, Logan, UT), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.43 mg/ml GlutaMAX-1, and penicillin-streptomycin (penicillin G sodium, 100 U/ml, and streptomycin sulfate, 0.10 mg/ml) (Invitrogen Life Technologies, Carlsbad, CA) at 37°C in 5% CO₂. Cells

were passaged with trypsin-EDTA (0.05% trypsin and 0.53 mM tetrasodium EDTA), and were used for experiments between passages 9 and 17 after initial isolation. IL-1 β and TNF- α were purchased from R&D Systems, Inc. (Minneapolis, MN). Fluticasone propionate was supplied by GlaxoSmithKline (Research Triangle Park, NC). *Streptomyces* HYAL was purchased from ICN Biomedical, Inc. (Aurora, OH), and poly I:C from Invivogen (San Diego, CA). The monocyte cell line, U937, was obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 (Invitrogen) with 10% FBS.

HLFs were seeded at 3.5×10^4 /well in 24-well plates in 10% FBS DMEM. After 24 hours, cells were growth arrested for 48 hours in 0.1% FBS DMEM, at which point the cells were approximately 80% confluent. Medium was then removed and cells were stimulated with agonists for 20 to 24 hours in fresh 10% or 0.1% FBS DMEM (10%, except where noted). The media and cell layers were assessed for hyaluronan levels, and parallel wells were used for cell counts. To assess the expression of ECM components, HLFs were seeded at 3.6×10^5 /60 mm dishes, and the above protocol for cell stimulation performed. At specific time points (6 and 12 h) after stimulation, total RNA was isolated, as described subsequently here.

Quantitative Real-Time RT-PCR

DNA-free RNA was obtained from cell culture monolayers with the Total RNA Isolation Kit from Agilent Technologies (Wilmington, DE), according to the manufacturer's directions. cDNA was prepared by reverse transcription with random primers with the High Capacity cDNA Archive Kit from Applied Biosystems Division (ABI), Perkin Elmer (Foster City, CA). PCR was performed with an ABI Prism 7900HT Sequence Detection System with TaqMan Fast Universal PCR Master Mix from ABI, as directed by the manufacturer. ABI gene expression assays used were as follows: hyaluronan synthase (HAS)-1 Hs00758053_m1; HAS-2 Hs00193435_m1; HAS-3 Hs00193436_m1; CD44 Hs00153304_m1; Hyal 1 Hs00537920_g1; Hyal 2 Hs00186841_m1; ADAMTS1 Hs00199608_m1; ADAMTS4 Hs00192708_m1; ADAMTS5 Hs00199841_m1; total versican Hs00171642_m1; versican V0 Hs01007944_m1; versican V1 Hs01007937_m1; versican V2 Hs01007943_m1; versican V3 Hs01007941_m1; 18S Hs99999901_s1; human TBP Hs99999910_m1. For each group, assays were run in duplicate on samples isolated from triplicate dishes. Normalized mRNA levels were then expressed as fold of levels in control cells by the comparative Ct method (Applied Biosystems), or as estimated copy numbers of mRNA for the versican isoforms and HAS1-3 by the relative standard curve method (ABI). Standard curves with R^2 greater than 0.995 were generated with versican PCR products greater than 500 bp spanning the versican isoform splice junctions and full-length cDNAs for HAS-1, -2, and -3. Target isoform quantitative PCR efficiency was not affected by the other isoforms, or by background nucleic acids.

Hyaluronan Enzyme-Linked Sorbent Assay

To isolate samples, media, and cell layers from cultures were digested with pronase (300 μ g/ml) in 0.5 M Tris (pH 6.5) for 18 hours at 37°C. To isolate hyaluronan from the cell layer, tissue culture dishes were rinsed with PBS and incubated in the pronase solution for 18 hours, scraped, and removed to Eppendorf tubes for storage. After digestion, the pronase was inactivated by heating to 100°C for 20 minutes. We used a modification (16) of a previously described (26) competitive enzyme-linked sorbent assay in which the samples to be assayed were first mixed with biotinylated hyaluronan binding protein (HABP, the N-terminal hyaluronan-binding region of aggrecan) (bPG) and then added to hyaluronan-coated microtiter plates, the final signal being inversely proportional to the level of hyaluronan added to the bPG.

Monocyte Adhesion Assay

HLFs were seeded in 96-well plates at 3×10^4 /cm² in 10% FBS DMEM. After 24 hours, the medium was changed to 0.1% FBS, and the cells were incubated for 48 hours. They were then incubated for 20 to 24 hours with fresh 10% FBS medium with either no addition, 1 ng/ml each IL-1 β and TNF- α , or 20 μ g/ml poly I:C. Before assay,

the human monocyte cell line, U937 ($10 \times 10^6/\text{ml}$), was incubated with calcein-AM (Invitrogen) ($0.5 \mu\text{g}/\text{ml}$) for 30–45 minutes, washed twice in RPMI medium (no phenol red), and resuspended to $3 \times 10^6/\text{ml}$. Some HLFs were treated with *Streptomyces* HYAL ($0.66 \text{ U}/\text{ml}$) for 20–30 minutes before the adhesion assay. Then, $100 \mu\text{l}$ of U937 cell suspension was added to the HLF monolayers and allowed to adhere for 90 minutes at 4°C (13). Plates were washed gently three times, and visualized to ensure monolayer integrity, before measuring the plate in a Fusion Series Universal Microplate Analyzer (Packard Bioscience Co., Meriden, CT), with excitation and detection wavelengths of 485 and 535 nm, respectively (27). To determine the effect of hyaluronan fragments or anti-versican antibodies on synthesis of the poly I:C-induced ECM, inhibitors were added in $50 \mu\text{l}$ of twice the final concentration, followed by $50 \mu\text{l}$ of twice the final concentration of poly I:C to control or poly I:C wells that had been arrested in low-serum medium. The reagents were removed at the end of the ECM formation period (20–24 h), when the incubation media were replaced with fresh RPMI medium containing calcein-AM-labeled monocytes for the binding phase of the experiment. LF99, an antibody to the N terminus of versican, was a generous gift from Dr. Larry Fisher, Craniofacial and Skeletal Disease Branch, National Institutes of Dental Research, National Institutes of Health (Bethesda, MD) (28). It was used at 2–10% of the medium volume. Antibody to the C terminus of versican, 2-B-1 (Seikagaku America, Falmouth, MA), was also added at $50 \mu\text{g}/\text{ml}$. Hyaluronan fragments (molecular weight, $<50 \text{ kD}$) were added at $30 \mu\text{g}/\text{ml}$. Concentrates of mouse IgG1 monoclonal antibodies to the hyaluronan-binding region of human versican (12C5), and the *Drosophila* protein, frizzled, were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development, and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA). These antibody concentrates were added at different concentrations up to 1% of the medium volume. Antibodies and hyaluronan fragments were also added to the monocyte-containing medium during the binding phase of the experiment. In addition to the hyaluronan fragments and anti-versican antibody, used in the same concentrations as above, an antibody to CD44 (monoclonal anti-human CD44 clone A3D8; Sigma, St. Louis, MO) was also used during the binding phase of the experiment at $20 \mu\text{g}/\text{ml}$ (13).

Immunocytochemistry

HLFs were cultured and treated on glass coverslips (4 cm^2), as described previously here, for hyaluronan analysis and/or monocyte adhesion assays. For visualization of hyaluronan and versican, cell layers were fixed in an acid alcohol formalin buffer, as described by Lin and colleagues (29), thereby allowing maximum retention of the hyaluronan and associated proteins. For visualization of monocyte binding to HLF cell layers, cells were fixed in -20°C methanol and allowed to air dry (9). The coverslips were blocked at room temperature with 1% BSA/5% normal donkey serum in PBS for at least 1 hour. Subsequently, they were incubated with combinations of bPG and 2-B-1, a mouse monoclonal antibody to the C terminus of versican (Seikagaku Corp., Tokyo, Japan) in 0.1% BSA/0.5% normal donkey serum in PBS overnight at 4°C . After three washes, cells were incubated with Texas red streptavidin ($2 \mu\text{g}/\text{ml}$), Alexa Fluor 488 donkey anti-mouse IgG ($8 \mu\text{g}/\text{ml}$), and 4',6'-diamidino-2-phenylindole ($1 \mu\text{g}/\text{ml}$) for 1 hour. After three washes in PBS, coverslips were mounted in Gel/Mount (Biomed, Foster City, CA), and photographed with an Axioskop fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY).

Western Analysis

For Western blotting, proteoglycans were digested by chondroitinase ABC lyase, applied to SDS-PAGE, and electrophoretically transferred to $0.2\text{-}\mu\text{m}$ nitrocellulose membranes (Schleicher and Schuell, Keene, NH) with a Bio-Rad Transblot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). The transferred proteins were then detected with the primary antibody to versican, 2-B-1, and enhanced chemiluminescence (Western-Light Chemiluminescent Detection System with CSPD proprietary luminescent substrate; Applied Biosystems, Foster City, CA). Alternatively, cell layer extracts and media were ethanol precipitated and dissolved in loading buffer before electrophoresis and transfer to

nitrocellulose. Transferred proteins containing the C-terminal epitope, DPEAAE, were detected with the versican V0/V1 Neo antibody (Affinity BioReagents, Golden, CO) and enhanced chemiluminescence (Super Signal West Dura Chemiluminescent Detection Kit; Pierce, Rockford, IL). In some experiments, to detect the epitope, DPEAAE, FBS was first diluted sevenfold with PBS and returned to its original concentration with three repetitions with an Amicon Centricon Plus-70 (Millipore, Billerica, MA), which excludes molecules with a hydrodynamic size of less than $100,000 \text{ Da}$. This process should reduce the concentration of smaller molecules, including BSA, to 3% of their original content. Within experiments, lanes were loaded with material derived from equal numbers of cells. Cell numbers were obtained by counting cells grown in parallel dishes for each treatment during each experiment.

Pulse-Chase Analysis of Hyaluronan Turnover in the ECM

Low-serum-arrested HLFs were stimulated for 24 hours in 10% FBS medium containing $40 \mu\text{Ci}/\text{ml}$ [^3H]-glucosamine, with or without poly I:C. The labeling medium was removed, and the cell layer washed gently once with serum-free medium. The cells were then incubated with fresh 0.1% serum medium and harvested along with the medium at different times up to 24 hours. Media and cell layers were digested with pronase ($300 \mu\text{g}/\text{ml}$) in 0.5 M Tris (pH 6.5) for 18 hours at 37°C , followed by heat inactivation at 100°C . The presence of tritium-labeled hyaluronan was determined by CPC precipitation of aliquots of the medium and cell layer extract with and without *Streptomyces* HYAL treatment, the difference between those two values being considered as labeled hyaluronan. Radioactivity remaining in the cell layer was expressed as a percent of that present at chase time zero.

Determination of Hydrodynamic Size of Hyaluronan

HLFs were labeled with [^3H]-glucosamine, with or without poly I:C, as described previously here. After 24 hours, cell layers were harvested with pronase and heat inactivated, as for hyaluronan enzyme-linked sorbent assays and passed over G-50 columns (GE Healthcare, Piscataway, NJ) to remove unincorporated label. Tritium-labeled glycosaminoglycans were applied to an S-1,000 (GE Healthcare) column to determine the hydrodynamic size of the radiolabeled hyaluronan. Samples were split, and replicates were digested with HYAL before passage over the S-1,000 to identify the HYAL-sensitive (hyaluronan) portion of the radioactivity.

Visualization and Quantitation of Pericellular Matrix

The existence of a pericellular coat has previously been operationally defined by the exclusion of fixed red blood cells (RBCs) (30). Pericellular matrix formation was quantitated morphometrically by point counting. For each condition, random phase-contrast digital images were captured on a Leica DM IRB inverted microscope (Leica, Wetzlar, Germany) with a SPOT Pursuit CCD camera (Diagnostic Instruments, Sterling Heights, MI). A 360-point grid was superimposed on digital images, and the number of hits on noncoat (RBCs included), coat (RBCs excluded), and cell was recorded. Cell area and coat area fractions were determined by the ratio of cell and coat point counts per total image area point counts.

Preparation of Hyaluronan Fragments

Hyaluronan (200 mg) was dissolved in 20 ml of 0.1 M ammonium acetate (pH 6.0), and digested for 72 hours with 50 U of *Streptomyces* HYAL. The enzyme was inactivated by boiling for 20 minutes, and the hyaluronan fragments were fractionated by ultrafiltration through Centricon microconcentrators (Millipore) with molecular weight cut-offs of 50 kD . Hyaluronan fragments passing through the Centricon 50 are designated hyaluronan less than 50 kD . Samples were lyophilized, resuspended in PBS, and filter sterilized. Uronic acid concentration was measured by the orcinol method.

Statistical Analysis

Statistical significance was determined by a two-tailed Student's *t* test.

Figure 1. Polyinosine-polycytidylic acid (Poly I:C) alters the distribution of versican and hyaluronan between medium and cell layer in human lung fibroblast (HLF) cultures. (A and B) Versican, (C) hyaluronan. (A) Cell layers from HLFs treated for 24 hours with medium containing 10% FBS with poly I:C, IL-1 β plus TNF- α , or no addition were harvested for Western blot analysis after digestion with chondroitin ABC lyase. Material from an equal number of cells was used in each lane, and represent a separate 100-mm dish. Lanes 1–3, control; lanes 4–6, IL-1 β plus TNF- α ; lanes 7–9, poly I:C. (B) Western blots showing versican retention in the cell layer of HLFs treated with poly I:C or IL-1 β plus TNF- α in 10% FBS medium (shown in A) or total culture extracts (medium plus cell layer; data not shown) were scanned and quantitated with NIH Image-J. Results were normalized to controls. Closed bars, poly I:C; open bars, IL-1 β plus TNF- α . (C) After 24-hour exposure to medium containing 10% FBS with poly I:C or IL-1 β plus TNF- α , total hyaluronan was increased in both treatments above control, but the amount of hyaluronan in cell layers was significantly increased only in poly I:C-treated cultures. Closed bars, medium; open bars, cell layer. * $P < 0.05$, ** $P < 0.01$ compared with control. All of the experiments represented in this figure were repeated three or more times with similar results.

RESULTS

Poly I:C Causes Accumulation of Versican and Hyaluronan in the ECM

Western blot analyses revealed an enrichment of two high-molecular weight bands at approximately 450 and 350 kD, corresponding to versican V0 and V1 (31, 32), in cell layer extracts of the poly I:C-treated cultures, with little versican present in similar extracts from control or cytokine-treated cultures (Figure 1A). No significant differences were found in the fold change of total versican (cell layer plus medium) of poly I:C-treated cells, whereas it was reduced in cytokine-treated cultures (Figure 1B). Poly I:C treatment of HLFs caused a significant increase in the hyaluronan content in the ECM and correspondingly reduced the amount of hyaluronan in the medium when compared with control or cytokine-treated cultures (Figure 1C). Significantly increased retention of hyaluronan and versican in the ECM of poly I:C-treated HLFs was confirmed in a line of cells from a second individual (data not shown). These results indicate that poly I:C has a major effect on the accumulation of versican and hyaluronan within the ECM of HLF cultures.

Poly I:C Promotes the Formation of a Pericellular Matrix

Colocalization of versican and hyaluronan in the cell layer has been associated with the accumulation of a viscous cell coat that can be visualized by particle exclusion. Because poly I:C causes a significant increase in hyaluronan and versican content of the ECM above that produced by cytokine-treated or untreated control cells, we used the particle exclusion assay to determine if poly I:C induced an increase in the total volume of the ECM coat. Indeed, poly I:C caused a significant increase in the volume of the pericellular matrix surrounding the HLF (Figure 2). There was no measurable increase in cell coat after treatment of HLF with IL-1 β plus TNF- α . This morphological estimate of increased cell coat recognizes changes in the area on the plane of the tissue culture dish. However, visual observations of piled up erythrocytes in the particle exclusion assay suggest that there may actually be a deeper layer of matrix in the poly I:C-treated cultures, resulting in an underestimate of the increase by our method (data not shown).

Expression and Degradation of Versican in HLF Cultures are Altered by Poly I:C

mRNA copy numbers for the versican splice variants, V0, V1, V2, and V3, were determined. Control cells contained approximately four times as many copies of V1 mRNA per copy of 18S RNA compared with V0, whereas V2 and V3 had very low copy numbers at approximately 0.8% the number of V1 copies. The mRNAs for splice variants V0 and V1 were significantly reduced in comparison to controls by treatment with either IL-1 β plus TNF- α or poly I:C (Figure 3). mRNAs for V2 and V3 were similarly decreased (data not shown). Therefore, increased accumulation of versican in the ECM of poly I:C-treated cultures is apparently not due to increased versican synthesis by the HLF.

Another possible explanation for the accumulation of versican in the ECM is a reduced rate of versican degradation. To assess this, we determined the accumulation of the epitope, DPEAAE, existing on the N-terminal degradation product of versican, when cleaved by ADAMTS1, -4, or -5 (22). This epitope was reduced in the ECM of poly I:C-treated cells (Figure 4A). Although the amount of this versican fragment was reproducibly low in poly I:C-treated cell layers, IL-1 β plus TNF- α -treated cells showed a more variable response, either

Figure 2. Particle exclusion assays show increased cell coat area in cells treated with poly I:C in comparison with control cells or those treated with IL-1 β plus TNF- α . HLFs were treated for 24 hours with 0.1% FBS medium alone, or 10% FBS alone, or 10% FBS containing either poly I:C, or IL-1 β plus TNF- α . (A) 0.1% FBS, (B) 10% FBS, (C) 10% FBS and IL-1 β plus TNF- α , (D) 10% FBS plus poly I:C. (E) Extracellular matrix (ECM) area in each condition, as defined by exclusion of fixed red blood cells, was quantitated. $**P < 0.01$ compared with poly I:C. This experiment was performed twice with similar results.

above or below control levels, but never as low as that found in cells treated with poly I:C (data not shown). Expression of ADAMTS1 and -5 was reduced by both treatments. Reduced levels of ADAMTS1 and -5 mRNA may help to explain the reduction in DPEAAE in poly I:C-treated cells. On the other hand, regulation may occur at the level of enzyme activation. ADAMTS4 mRNA was greatly increased in IL-1 β plus TNF- α -treated cells (12-fold), with a slight increase in poly I:C-treated cells (nearly twofold) (Figure 4B). This dramatic difference in ADAMTS4 induction (between poly I:C and IL-1 β plus TNF- α) may help to explain the absence of versican from the IL-1 β plus TNF- α -induced ECM.

Poly I:C Causes Retardation of Hyaluronan Turnover

To determine if poly I:C influences the synthesis of hyaluronan by HLF, we evaluated the expression of mRNA transcripts for the three hyaluronan synthases (HAS-1, -2, and -3) by quantitative RT-PCR. Copy numbers for the three HAS mRNAs were determined, and HAS-2 was found to be the predominant isoform in HLF control cultures. Copy numbers for HAS-2 were at least four orders of magnitude greater than for HAS-1, and two orders of magnitude greater than for HAS-3 (data not shown). Treatment of these cells with poly I:C, as well as with IL-1 β plus TNF- α , raised the levels of HAS-1 mRNA significantly when compared with nontreated control cultures (Figure 5A). The increase was greatest in IL-1 β plus TNF- α -treated

Figure 3. Estimated copy numbers for the versican splice variants, V0 and V1 are not increased by poly I:C. mRNA for each splice variant was normalized to abundance of 18S RNA in HLFs treated for 12 hours with control, IL-1 β plus TNF- α , and poly I:C. Values for both treatments were reduced in comparison with controls. $**P < 0.01$ compared with control. This experiment was performed twice with similar results. In addition, a significant reduction in the mRNA level for total versican was found in both IL-1 β plus TNF- α , and poly I:C-treated cells at 6 and 12 hours after treatment in three different experiments (data not shown).

cells where ECM deposits of hyaluronan were not significantly increased (see Figure 1). Another difference was that HAS-2 mRNA levels were decreased by poly I:C treatment, whereas they were not changed by cytokine treatment when compared with controls. Because the HAS-2 mRNA was substantially more abundant than mRNA for the other HAS enzymes, poly I:C caused a reduction in the total amount of HAS mRNA. In addition, cytokine treatment elevated HAS-3 mRNA levels over controls, whereas poly I:C had no effect on the expression of this isoenzyme (Figure 5A). Collectively, these results indicate that HAS-1 activity may be required for the increased accumulation of hyaluronan and versican in the ECM of the HLF cultures, but that HAS-1 expression alone is not sufficient, and some other event must occur.

Hyaluronan produced in the presence of poly I:C is qualitatively different from controls. ECM hyaluronan metabolically labeled with tritiated glucosamine during incubation with poly I:C in 10% serum medium has a greater hydrodynamic size than that isolated from control cultures with 10% serum alone (Figure 5B). Although there was some high-molecular weight hyaluronan, which elutes in the void volume in the control cultures, the percentage of high-molecular weight hyaluronan was greater in cultures treated with poly I:C. In control and poly I:C-treated cells, all of the large [³H]-glucosamine-labeled material, which eluted at $K_{av} \leq 0.6$, was degraded by HYAL (not shown).

Figure 4. (A) Treatment with poly I:C reduces the accumulation of a versican degradation product in the ECM. After 24 hours of treatment with control medium, or poly I:C in medium containing 10% FBS concentrate (hydrodynamic size, >100,000 Da), cell layer proteins were harvested with urea buffer and ethanol precipitated before Western blotting with an 8% PAGE gel with antibody to DPEAAE, the C-terminal epitope on versican fragments produced by degradation by a disintegrin-like and metalloproteinase with thrombospondin type 1 motif (ADAMTS) 1, 4, or 5. Material from an equal number of cells was used in each lane, and represent a separate 100-mm dish. (B) mRNAs for versican-degrading enzymes were altered by both poly I:C and IL-1 β plus TNF- α . At 6 hours after treatment, levels of mRNA for ADAMTS 1 and 5 were similarly reduced by both treatments. ADAMTS 4 mRNA was only slightly changed by poly I:C treatment, whereas it was increased 12-fold in IL-1 β plus TNF- α -treated cells. Up-regulation of mRNA for ADAMTS 4, a versicanase, may help to explain the absence of versican in IL-1 β plus TNF- α -treated cell layers. Closed bars, poly I:C; open bars, IL-1 β plus TNF- α . * P < 0.05, ** P < 0.01 compared with control. All of the experiments shown in this figure were performed at least three times with similar results. Similar changes in mRNA were obtained at 12 hours after treatment.

Increased hyaluronan accumulation in the ECM could be the result of decreased turnover. To examine this possibility, we performed pulse-chase experiments in which control, IL-1 β plus TNF- α , or poly I:C-treated cells were simultaneously incubated with [3 H]-labeled glucosamine and then chased in 0.1% FBS-containing medium and evaluated for hyaluronan loss. Control cells displayed an early, rapid decline in radio-labeled hyaluronan, followed by a more gradual second rate of

loss. In the poly I:C-treated cultures, ECM released less radio-labeled hyaluronan during the early 0- to 6-hour decay, whereas loss of label during the second, 6- to 24-hour phase, occurred at a rate similar to that of control cultures. IL-1 β plus TNF- α -treated cells had an initial rate similar to that of controls. Values at later times were not determined for that treatment (Figure 6A). Very large hyaluronan (eluting in the void volume of the column) has been shown to resist degradation when added exogenously to cultures (33). Therefore, the larger hydrodynamic size of hyaluronan, which accumulated in the ECM of poly I:C-treated cultures (Figure 5B), may partially explain the slower rate of turnover of ECM hyaluronan.

Possible mechanisms for reduced degradation of ECM hyaluronan produced in response to poly I:C include: reduced production of enzymes that degrade hyaluronan (the HYALS), and/or reduced expression of the cell surface receptor, CD44, which is required for internalization and degradation of exogenous hyaluronan (24). mRNAs for HYAL 1 and 2 were significantly down-regulated in HLFs (over fourfold) 6 hours after the addition of poly I:C (Figure 6B). These mRNAs were also reduced, although to a lesser extent, in IL-1 β plus TNF- α -treated cells, where hyaluronan was not retained in the ECM. Thus, regulation of these enzymes may not be sufficient to explain the regulation of hyaluronan accumulation. In contrast, mRNA for CD44 was down-regulated in poly I:C-treated cells, but increased in IL-1 β plus TNF- α -treated cultures when compared with controls (Figure 6B). This suggests that reduction in CD44 expression may be an important factor in accumulation of hyaluronan in the ECM of these cultures.

Versican Is Absent in ECMs Generated by HLFs in Response to IL-1 β plus TNF- α , but Enriched in ECMs in Response to Poly I:C

To further evaluate if the presence or absence of versican typified the difference between the ECMs produced by HLFs stimulated with poly I:C or IL-1 β plus TNF- α , we examined hyaluronan and versican accumulation in the pericellular matrix of control, poly I:C-, and IL-1 β plus TNF- α -treated cells by fluorescent staining with biotinylated HABP (bPG) and 2-B-1, a monoclonal antibody to versican (Figures 7A–7I). There was increased hyaluronan staining of fibrillar-like ECM structures in poly I:C-treated cultures compared with the cytokine-treated cultures and untreated controls (Figures 7A, 7D, and 7G). In addition, poly I:C treatment of HLFs caused an enrichment of versican staining in the same fibrillar pattern as that observed for hyaluronan staining, and showed colocalization when the images were merged (Figures 7G–7I). On the other hand, extracellular versican staining was conspicuously absent in the cytokine-treated and control cultures (Figures 7B and 7E).

Monocyte Adhesion in Poly I:C-Treated Cultures Can Be Blocked by Antibodies to Versican

To test whether the versican-enriched ECM made by HLFs in the presence of poly I:C produced a matrix that supported monocyte adhesion, U937 monocytes were added to the HLF cultures 24 hours after stimulation, as previously described (27). Poly I:C caused a dramatically increased hyaluronan-dependent monocyte adhesion to the HLF cultures over control values, whereas IL-1 β plus TNF- α stimulation failed to cause an increase in monocyte retention (Figure 8A). Significantly increased monocyte adhesion after poly I:C treatment was also found in experiments with HLFs from a second individual (data not shown). Addition of a monoclonal antibody concentrate specific for the N terminus of versican, 12C5, during matrix

Figure 5. (A) Expression of the HAS genes is differently altered by IL-1 β plus TNF- α and poly I:C. Levels of mRNA for the hyaluronan synthases, HAS-1 through -3, were determined by quantitative RT-PCR and expressed for each treatment as fold increase above control at 12 hours after addition of treatments. mRNA levels for HAS-1 were increased in both IL-1 β plus TNF- α - and poly I:C-treated cells. HAS-2 mRNA levels were decreased in poly I:C-treated cells. HAS-3 mRNA was increased only in the IL-1 β plus TNF- α -treated cells. Open bars, IL-1 β plus TNF- α ; closed bars, poly I:C. ** P < 0.01 compared with control. This experiment was performed four times with similar results, and significant similar changes were found at 6 hours after treatment. (B) Treatment with poly I:C increases the average hydrodynamic size of hyaluronan in the ECM secreted by HLFs. HLFs were metabolically labeled with tritiated glucosamine for 24 hours in the presence of 10% serum-containing medium alone or with addition of 20 μ g/ml poly I:C. Radiolabeled cell layer extracts were separated from free glucosamine with a Sephacel G-50 (GE Healthcare) column, and then applied to an S-1,000 column (GE Healthcare) in 4 M guanidine buffer to determine the hydrodynamic size profile. Open circles, poly I:C; closed circles, control. All fractions above a K_{av} of 0.6 were completely degraded by hyaluronidase (HYAL) (data not shown). Thus, all of the high-molecular weight material eluted from this column is hyaluronan. This experiment was performed twice with similar results.

formation, blocked the poly I:C-induced and HYAL-sensitive binding of monocytes to HLFs, whereas a control concentrate of an irrelevant monoclonal antibody (1C11) did not (Figure 8B). The monoclonal concentrates contained similar concentrations of mouse IgG with 145 μ g/ml in 12C5 and 171 μ g/ml in 1C11. LF99 (28), a rabbit antibody against the N terminus of versican, also inhibited monocyte binding, but a negative control was not available (data not shown). In addition, the antibody to the C terminus of versican, 2-B-1, had no effect (data not shown).

The requirement of hyaluronan for monocyte adhesion is strongly implied by its sensitivity to HYAL. In addition, we directly blocked the formation of the ECM complex by coincubating the poly I:C-stimulated cells with hyaluronan fragments (molecular weight, $\leq$ 50,000 Da), which have been shown to compete with high molecular weight hyaluronan in

Figure 6. (A) Pulse-chase analysis of [³H]-labeled hyaluronan shows reduced turnover in poly I:C-treated cells. HLFs were metabolically labeled with tritiated glucosamine for 24 hours in the presence of 10% serum-containing medium alone or with addition of IL-1 β plus TNF- α or 20 μ g/ml poly I:C, as in Figure 5B, and then chased with fresh 0.1% FBS medium. Remaining radioactivity was normalized to the amount in the cell layers at time zero of the chase period. Closed circles, controls; open circles, poly I:C; open triangles, IL-1 β plus TNF- α . The initial rate of degradation was much greater in controls and in IL-1 β plus TNF- α - than in poly I:C-treated cells, whereas a second, more gradual, rate appeared to be similar for both control and poly I:C-treated cells. This experiment was performed twice with similar results. (B) mRNAs associated with hyaluronan degradation are decreased by poly I:C treatment. Cells were arrested in low-serum-containing medium, and then stimulated with IL-1 β plus TNF- α , poly I:C, or no addition in 10% serum-containing medium. The cells then were harvested for mRNA analysis after 6-hour incubation. CD44, HYAL1, and HYAL2 mRNAs were significantly decreased in poly I:C-treated cells in comparison with controls. There was also a small, but significant, decrease in HYAL2 mRNA in IL-1 β plus TNF- α -treated cells. Reduction in these degradative enzymes and in CD44, the cell surface receptor for hyaluronan, may help to explain the slower release of radiolabeled hyaluronan from poly I:C-treated cells. In contrast, mRNA for CD44 was increased over twofold in IL-1 β plus TNF- α -treated cells. Open bars, IL-1 β plus TNF- α ; closed bars, poly I:C. * P < 0.05, ** P < 0.01 compared with control. This experiment was performed three times with similar results.

other systems. These fragments completely blocked the effect of poly I:C on monocyte adhesion when added during the formation of the ECM (Figure 9A). Although addition of hyaluronan fragments and anti-versican antibody completely prevented the formation of a monocyte-retaining ECM when added during

Figure 7. Poly I:C increases colocalization of versican and hyaluronan in the cell layer. Cells from HLF cultures treated for 24 hours with 10% FBS-containing medium with poly I:C, IL-1 β plus TNF- α , or no addition (control) were fixed and stained for hyaluronan (red), versican (green), or nuclei (blue). Compared with controls, increased amounts of hyaluronan were present in both IL-1 β plus TNF- α - and poly I:C-treated cells, whereas versican was increased in only poly I:C-treated cells. When the two stains were merged in a single exposure, colocalization (yellow) was present on control cells and those treated with poly I:C. (A–C) Control, (D–F) IL-1 β plus TNF- α , (G–I) poly I:C. (A, D, and G) Hyaluronan, (B, E, and H) versican, (C, F, and I) merged images with both stains (in I, nuclei appear magenta due to the presence of red hyaluronan stain above the plane of the cell). Monochrome images of the blue (4',6'-diamidino-2-phenylindole [DAPI], nuclear), red (Texas red, hyaluronan), and green (Alexa Fluor 488, versican) emissions were collected at equivalent camera settings (exposure time and γ) for each color with a SPOT cooled CCD digital camera. Images were pseudocolored with the SPOT software, and merged with Adobe Photoshop (San Jose, CA). The brightness and contrast from all images of control and treated samples were adjusted equally for each color. This experiment was performed four times with similar results.

stimulation, these substances had a lesser effect when added during the 90-minute monocyte incubation phase of the experiment (data not shown). In additional studies, we added a blocking antibody to CD44, the monocyte cell surface receptor for hyaluronan, during the monocyte incubation period. This treatment inhibited monocyte adhesion by 50% compared with controls (data not shown), which is consistent with the findings of de la Motte and colleagues (13), who were able to obtain approximately 50% reduction of leukocyte binding to poly I:C-stimulated intestinal smooth muscle cells by addition of anti-CD44 antibody.

We have previously demonstrated that the steroid, fluticasone, can inhibit hyaluronan synthesis by HLFs grown in low-serum medium (16). To test whether this anti-inflammatory agent could affect the production of a monocyte-retaining ECM, fluticasone was added to HLFs treated with poly I:C in 10% FBS medium. The drug caused a significant ($P < 0.05$) inhibition of hyaluronan accumulation in the cell layer at 10^{-9} M. This result was mirrored by a similar significant reduction in

Figure 8. Inhibition of monocyte retention by interference with ECM formation in poly I:C-treated cells. (A) Poly I:C increases monocyte adhesion to HLF cultures, whereas IL-1 β plus TNF- α does not. Fluorescently labeled U937 monocytes were incubated with HLF cultures for 90 minutes at 4°C after 24-hour exposure of the HLFs to 10% FBS-containing medium with poly I:C (PIC), IL-1 β plus TNF- α , or no addition. Some wells were treated with HYAL for 20–30 minutes before addition of monocytes. Open bars, plus HYAL. This experiment was performed four times with similar results. ** $P < 0.01$ compared with control. (B) Formation of a poly I:C-induced monocyte-retaining ECM can be blocked by the addition of antibody to versican. HLFs were stimulated with poly I:C in 10% FBS-containing medium for 24 hours in the presence or absence of antibody to the N terminus of versican or a control antibody. The subsequent monocyte-binding assay was performed in the absence of antibody. Significant inhibition of monocyte retention by the ECM was observed. Monocyte binding was measured as arbitrary fluorescence units, reflecting Calcein AM in the cells. Fluorescence was linearly associated with monocyte number (data not shown). ** $P < 0.01$ compared with cells treated with poly I:C alone, ## $P < 0.01$ compared with cells treated with the same concentration of anti-versican antibody. Open bars, plus HYAL, gradient bars, anti-versican antibody (12C5), gray bars, mouse antibody against *Drosophila* frizzled protein (1C11) used as a control. This experiment was performed twice with similar results. “%AB” refers to concentration of monoclonal antibody concentrate by volume.

monocyte adhesion to the ECM of fluticasone-treated HLFs. Furthermore, Figure 9B demonstrates that there is a linear relationship between the percent inhibition of hyaluronan synthesis and HYAL-sensitive leukocyte binding, suggesting that fluticasone inhibition of monocyte retention is mediated by a reduction in hyaluronan synthesis.

To identify the site of interaction of the monocytes with the ECM of these cultures, the cultures with attached monocytes were fixed and immunostained for hyaluronan and versican. As shown in Figures 10A–10C, distinct hyaluronan- and versican-positive fibrillar or cable-like structures were present in the

Figure 9. Hyaluronan (HA) incorporation into the ECM is required for monocyte adhesion in poly I:C treated cells. (A) Inhibition of poly I:C-induced ECM formation by hyaluronan fragments (F). HLFs were stimulated with poly I:C in 10% FBS-containing medium for 24 hours in the presence or absence of low-molecular weight hyaluronan fragments. The subsequent monocyte-binding assay was performed in the absence of hyaluronan fragments. Some wells were treated with HYAL for 20–30 minutes before addition of monocytes. Complete inhibition of HYAL-sensitive monocyte binding was observed in the fragment-treated wells. Open bars, plus HYAL. * $P < 0.05$ compared with poly I:C; ** $P < 0.01$ compared with controls. Monocyte binding was measured as arbitrary fluorescence units, reflecting Calcein AM in the cells. Fluorescence was linearly associated with monocyte number (data not shown). This experiment was performed three times with similar results. (B) Fluticasone partially inhibits poly I:C-induced hyaluronan synthesis and poly I:C-induced formation of a monocyte-retaining ECM. HLFs were treated with poly I:C for 24 hours in the presence of different concentrations of fluticasone (0 , 10^{-9} , 10^{-8} , and 10^{-7} M), and then assayed for hyaluronan synthesis or for monocyte adhesion. In both cases, the results were significantly reduced from control levels ($P < 0.05$) at 10^{-9} M fluticasone. When the degree of inhibition of hyaluronan synthesis was plotted against the inhibition of monocyte retention, the relationship was linear. This experiment was performed twice with similar results.

ECM of the poly I:C-treated cultures, with monocytes clustered onto these structures (Figure 10D). Colocalized versican and hyaluronan can be seen in the extracellular spaces between the clustered monocytes. In contrast, in the control and IL-1 β plus TNF- α -treated cultures, the monocytes were sparsely adherent as single cells at apparently random locations on the cell surface and on the bare plastic dish (data not shown).

DISCUSSION

We have shown that the viral mimetic, poly I:C, promotes the formation of an ECM by HLFs that is enriched in hyaluronan and versican. Although a similar effect of poly I:C has been

Figure 10. Colocalization of adherent monocytes with versican and hyaluronan in poly I:C-induced ECM. Cells treated as in Figure 7 were also incubated with monocytes for 90 minutes at 4°C before fixation. The monocyte nuclei were also labeled with DAPI (blue). No HLF nuclei are visible in Figures 8A–8D. (A) Hyaluronan, (B) versican, (C) merged, and (D) C plus monocytes. Monocyte aggregates were predominantly located where versican and hyaluronan were colocalized. Fluorescent micrograph images of the blue (DAPI, nuclear), red (Texas Red, hyaluronan), and green (Alexa Fluor 488, versican) emissions were collected with a Nikon Coolpix digital camera (Nikon, Melville, NY). Brightness and contrast were identical for each color in single and merged images. This experiment was performed twice with similar results.

demonstrated in other mesenchymal cells, such as intestinal smooth muscle cells (9, 13), we have gone on to show that poly I:C primarily affects the turnover of versican and hyaluronan, and does not require increased synthesis of these macromolecules. Furthermore, we showed that poly I:C affects the expression of enzymes that degrade both versican and hyaluronan. In addition, we demonstrated that versican is a key component in the binding of monocytes to this ECM, as ECMs enriched in hyaluronan, but not versican, do not bind monocytes, and that blocking antibodies to versican block the formation of ECMs enriched in both versican and hyaluronan to which monocytes adhere.

Typically, growth factors and cytokines regulate versican and hyaluronan by altering the mRNA levels for versican and HAS (34–36). In contrast, poly I:C increases the versican and hyaluronan content of the ECM by reducing the loss of these components from the ECM. Total versican (V0 and V1) in poly I:C-treated cultures did not increase, consistent with a reduction in mRNA for the three versican splice variants. On the other hand, retention of versican in the cell layer was complemented by a reduction in DPEAAE levels in the poly I:C-treated cell layers, indicating reduced versican degradation. mRNAs for versicanases, such as ADAMTS 1 and 5, were reduced in both the cytokine- and poly I:C-treated cells to a similar degree. However, mRNA for another versicanase, ADAMTS 4 (22), was greatly increased over controls in the cytokine-treated cells, and only slightly increased in the poly I:C-treated cells. Although this difference may be responsible for the lower amount of DPEAAE versican fragment in poly I:C-treated cells, it is also possible that furin or other ADAMTS processing enzymes are regulated differently by poly I:C. Reduced versican in IL-1 β plus TNF- α -treated cells, as well as a 12-fold increase in ADAMTS 4 mRNA, are consistent with other studies. For example, down-regulation of versican synthesis and mRNA levels after IL-1 β stimulation has been demonstrated in smooth muscle cells and in gingival fibroblasts (17). In addition, ADAMTS4 and aggrecanase activity were increased in articular

cartilage treated with IL-1 β plus TNF- α (37). ADAMTSs have been shown to regulate the formation of a viscous matrix, which is dependent upon the presence of brevican, another proteoglycan that cross-links hyaluronan, where degradation of brevican by ADAMTS4 reduces the viscosity of the ECM and allows increased extension of primary neurites (38). It is also possible that poly I:C regulates the expression of other versicanases, which include matrix metalloproteinases 1, 2, 3, and 7–9, and plasmin (39).

Although there was a modest increase in total hyaluronan accumulation in response to poly I:C, approximately 50% of the hyaluronan was deposited in the ECM, with the remaining 50% present in the culture medium. A similar effect of poly I:C on the distribution of hyaluronan among medium and ECM has been reported for arterial (40) and airway (41) smooth muscle cells. This is in sharp contrast to the distribution of hyaluronan in cytokine-treated cultures and untreated control cultures, where the bulk of the hyaluronan (~90%) was present in the culture medium. Thus, it appears that, in cultured HLFs, retention of versican and hyaluronan, rather than increased synthesis, is the predominant factor in production of this ECM. Furthermore, the HAS-1 enzyme may be required for monocyte retention by the ECM, as HAS-1 is the only isoform the mRNA level of which was increased above control by poly I:C. This is consistent with our findings with aortic smooth muscle cells, in which HAS-1 overexpression caused a significant increase in HYAL-sensitive monocyte adhesion to the ECM, whereas HAS-2 and -3 were less effective (27).

Retention of hyaluronan in the ECM is prolonged in cells treated with poly I:C in comparison to high-serum medium alone. Degradation of hyaluronan may be reduced due to lower expression of the HYALS 1 and 2, or to a reduction in CD44, which is required for degradation of exogenous hyaluronan by both HYALS (24). Cross-linking of hyaluronan by versican or other ECM components may interfere with hyaluronan internalization, and may reduce its availability to degradative enzymes. Udabage and colleagues (42) have shown that inhibition of HYAL activity in cultured breast cancer cells results in increased retention of hyaluronan in the cell layer. Hyaluronidase activity may be inhibited by the presence of inter- α -trypsin inhibitor (I α I), a component of the poly I:C-induced cross-linked hyaluronan matrix (9). I α I contains Kunitz-type inhibitor activity, and has been identified as a circulating form of HYAL inhibitor (43).

The composition of the poly I:C-induced ECM has not been completely defined. In studies of poly I:C-treated smooth muscle cell ECM, de la Motte and others have demonstrated the presence of hyaluronan, versican, I α I, and TNF-stimulated gene 6 (TSG6) (9, 13, 14). This ECM might also be reasonably expected to contain tenascin-C, link proteins 1 or 3, p-selectin glycoprotein ligand-1, and/or thrombospondin-1, which can contribute to cross-linking of the matrix (9, 14, 44, 45). In addition, it is not clear to what extent the accumulation of an expanded cell coat contributes to the formation of leukocyte-binding hyaluronan “cables.” Preferential binding of monocytes to hyaluronan cables has been demonstrated in smooth muscle cells (9, 13, 27). Selbi and colleagues (46) have shown the formation of a hyaluronan-dependent, monocyte-retaining ECM by proximal tubular cells of the kidney in response to bone morphogenic protein-7. In that study, monocyte binding depended upon the presence of hyaluronan-containing cables, which were absent from control cell cultures, even though similar amounts of hyaluronan were present in the control cell layers. In that system, where low serum was used, expression of HAS-2 was increased by bone morphogenic protein-7 compared with control. In contrast, our HLFs, which were treated

with poly I:C in high-serum medium, reduced HAS-2 mRNA in comparison with controls, whereas both systems demonstrated a similar reduction in expression of HYAL1 and -2. This suggests that poly I:C regulation of hyaluronan synthesis may vary with cell type or culture conditions, whereas the formation of a monocyte-retaining ECM is unaltered, resulting from consistent effects on versican and hyaluronan turnover.

The failure of monocytes to bind tightly to the ECM of control or IL-1 β plus TNF- α -treated cells may be due to the absence of versican. Versican is a ubiquitous ECM component that is important for cell adhesion, proliferation, migration, and angiogenesis (21, 36, 47, 48). It binds to many ECM components, including type I collagen, fibronectin, tenascin-R and -C, fibrillin, and fibulin-1 and -2 (49). Versican has been implicated in the formation of leukocyte aggregates through binding of its glycosaminoglycan chains to the C terminus of p-selectin glycoprotein ligand-1 (45), whereas, in poly I:C-stimulated smooth muscle cells, versican binds to thrombospondin-1 via its N-terminal region (44). Previous studies have also shown that aggregating proteoglycans, including versican, increase the viscosity of hyaluronan solutions. These proteoglycans stiffen the network of hyaluronan by reducing the length of the hyaluronan chains, and the porosity of the resulting ECM is decreased (50). This may explain the increased cell coat volume found in poly I:C-treated HLFs. It is possible that an increase in versican, together with any cross-linking that might occur, increases the viscosity of the pericellular matrix enough to withstand the washing procedures of the binding assay, thus increasing monocyte binding.

During the binding phase of the experiments, monocytes may be partially able to bind to the ECM without direct interaction with versican. This may be through monocyte CD44 binding to ECM hyaluronan, as has been demonstrated by de la Motte and colleagues (13). Anti-versican antibody caused partial inhibition of monocyte binding in our poly I:C-treated cells. This may be due to versican interactions with serglycin on the monocyte surface, as suggested by Day and de la Motte (14). Versican synthesis has been induced in peripheral monocytes in myocardial infarction and in culture by granulocyte-macrophage colony-stimulating factor (51). This could contribute to monocyte binding, or block it by interfering with monocyte binding to versican in the ECM.

Monocyte binding was reduced through interference with ECM formation either by using anti-versican antibody, or by using the steroid, fluticasone, which blocks hyaluronan synthesis. The effect of fluticasone is consistent with the possibility that part of its antiasthmatic activity is due to its ability to interfere with the generation of a proinflammatory ECM. Recently, dexamethasone has been shown to interfere with Toll-like receptor-3 signaling by blocking phosphorylation of IFN response factor 3 (52).

These studies indicate that double-stranded RNA can stimulate mesenchymal cells to accumulate an ECM by interfering with the turnover of specific ECM components, such as versican and hyaluronan, and that these ECMs serve as ligands for monocytes and other inflammatory cells. These observations raise the possibility that viruses may accelerate inflammation within the lung by promoting a proinflammatory microenvironment.

Some of the data contained in this article was also presented as a poster at the 2008 meeting of the American Academy of Allergy, Asthma, and Immunology (53).

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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