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Research Article

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Cytokine Regulation of Human Lung Fibroblast Hyaluronan (Hyaluronic Acid) Production

Evidence for Cytokine-regulated Hyaluronan (Hyaluronic Acid) Degradation and Human Lung Fibroblast-derived Hyaluronidase

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Abstract

We characterized the mechanisms by which recombinant (r) tumor necrosis factor (TNF), IFN-γ, and IL-1, alone and in combination, regulate human lung fibroblast hyaluronic acid (HA) production. Each cytokine stimulated fibroblast HA production. The combination of rTNF and rIFN-γ resulted in a synergistic increase in the production of high molecular weight HA. This was due to a synergistic increase in hyaluronate synthetase activity and a simultaneous decrease in HA degradation. In contrast, when rTNF and rIL-1 were combined, an additive increase in low molecular weight HA was noted. This was due to a synergistic increase in hyaluronate synthetase activity and a simultaneous increase in HA degradation. Human lung fibroblasts contained a hyaluronidase that, at pH 3.7, depolymerized high molecular weight HA to 10–40 kD end products of digestion. However, hyaluronidase activity did not correlate with fibroblast HA degradation. Instead, HA degradation correlated with fibroblast-HA binding, which was increased by rIL-1 plus rTNF and decreased by rIFN-γ plus rTNF. Recombinant IL-1 and rTNF weakly stimulated and rIL-1 and rTNF in combination further augmented the levels of CD44 mRNA in lung fibroblasts. In contrast, rIFN-γ did not significantly alter the levels of CD44 mRNA in unstimulated or rTNF stimulated cells. These studies demonstrate that rIL-1, rTNF, and rIFN-γ have complex effects on biosynthesis and degradation which alter the quantity and molecular weight of the HA produced by lung fibroblasts. They also show that fibroblast HA degradation is mediated by a previously unrecognized lysosomal-type hyaluronidase whose function may be regulated by altering fibroblast-HA binding. Lastly, they suggest that the CD44 HA receptor may be involved in this process. (*J. Clin. Invest.* 1992. 90:1492–1503.) Key words: interleukin 1 • tumor necrosis factor • γ-interferon • CD44 • lung

The first-author contributions of P. M. Sampson and C. L. Rochester were concurrent.

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Introduction

Hyaluronic acid (HA)[1] is a ubiquitous polysaccharide, which in vivo is usually a high molecular mass (10^6 to 10^7 D) component of extracellular matrix and biologic fluids. In these locations HA provides cellular support and regulates cell–cell adhesion, cellular spatial orientation, migration, proliferation, and differentiation (1, 2, 3–5), and the movement of interstitial fluid and macromolecules (6, 7). As a result, it is an important contributor to and regulator of inflammation (8), wound healing (9, 10), tissue remodeling (10, 11), and morphogenesis (12).

HA accumulation is regulated by the balance of biosynthesis and degradation. In normals this balance is finely controlled, maintaining appropriate tissue and serum HA levels. However, this balance is altered in many disease states. Increased amounts of HA have been noted in tissues surrounding malignancies (13), the serum of patients with liver disease (14), the serum and skin of patients with scleroderma (15, 16), and the serum and joint fluid of patients with rheumatoid arthritis (17, 18). Exaggerated levels of HA are also seen during compensatory lung growth (19), in the tissues of animals with bleomycin-induced pulmonary fibrosis (20), in the pleural space of patients with mesothelioma (21), and in the lavage fluid of patients with sarcoidosis (22), hypersensitivity pneumonitis (23), cystic fibrosis (24), alveolar proteinosis (25), and asthma (26). However, the mechanisms responsible for these HA abnormalities are poorly understood. This is due, in large part, to an incomplete understanding of the processes regulating HA metabolism in normals and the functioning of these pathways in disease states.

Hyaluronidases (HAases) are endohexosaminidases that initiate the degradation of high molecular weight HA (27, 28). The lysosomal-type HAases are widely distributed and play a major role in tissue and serum HA turnover (3, 27–31). Unlike the testicular HAases, they function best between pH 3 and 4 and are largely inactive at physiologic pH. This has led to the consensus that cellular HA catabolism requires HA-cell binding which is largely receptor-mediated and HA internalization (27, 28, 31). However, the processes that regulate cellular HAase activity and the effect of alterations in HAase activity on tissue HA accumulation are poorly understood. In addition, since normal human fibroblasts have been reported to lack

1. *Abbreviations used in this paper:* CPC, cetylpyridinium chloride; GAG, glycosaminoglycan; HA, hyaluronic acid; HAase, hyaluronidase; r, recombinant; TNF, tumor necrosis factor.

HAases (31, 32), the role of fibroblast-derived HAase in tissue repair and remodeling in humans has not been analyzed.

Studies from this and other laboratories have shown that a variety of inflammatory cytokines including IFN-γ, tumor necrosis factor (TNF), IL-1, and transforming growth factor-β regulate fibroblast HA production (33–35). The effects of some of these agents are, at least partially, mediated by cytokine-induced alterations in HA biosynthesis (33). The degree to which these effects are mediated by alterations in HA degradation, however, has not been elucidated. In addition, even though cytokine–cytokine interactions play a key role in biologic homeostasis and fibroblasts can produce HA with a range of molecular weights and biologic properties (18, 36–38), the ability of cytokines to interact in regulating the size as well as the quantity of HA produced by stromal tissues has not been adequately investigated.

To further understand the cytokine network regulating HA production, we characterized the effects of recombinant (r) IFN-γ, rTNF, and rIL-1, alone and in combination, on the size and quantity of HA produced by human lung fibroblasts. We also determined if the effects that were seen were due to alterations in HA biosynthesis or HA degradation and characterized the mechanism of the HA degradation that was noted.

Methods

Cytokines and antibodies

Human rIFN-γ (1.4×10^8 IU/mg protein) was kindly supplied by Hoffmann-LaRoche, Inc. (Nutley, NJ), rTNF (5×10^7 U/mg protein) was kindly supplied by Dr. H. Michael Sheppard (Genentech, Inc., South San Francisco, CA), and rIL-1-α was a generous gift of Dr. Peter Lomedico (Hoffmann-LaRoche, Inc.). These cytokines were added directly to fibroblast cultures after dilution. A monoclonal antibody against rIFN-γ and a monoclonal antibody against rTNF were obtained from Genentech, Inc. Rabbit antiserum against rIL-1-α was obtained from Smith, Kline & French, Inc. (King of Prussia, PA).

Fibroblast cell lines

Strain CCL-202 normal adult human lung fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and passed less than five times in our laboratory. They were grown to confluence in Dulbecco's modified Eagle's medium supplemented with nonessential amino acids (Gibco BRL, Grand Island, NY), penicillin, streptomycin (complete medium), and 10% heat-inactivated FBS (Gibco BRL).

Assessment of fibroblast glycosaminoglycan (GAG) biosynthesis

The GAG production of unstimulated and cytokine-stimulated confluent fibroblasts incubated under serum-free conditions was evaluated using isotopic and chemical approaches. The isotopic technique assessed fibroblast incorporation of [6-H^3]D-glucosamine hydrochloride into pronase-resistant, cetylpyridinium chloride (CPC)-precipitable material. In chosen experiments cell number was simultaneously assessed via hemacytometer counting. The chemical assessment employed pronase and nuclease treatment, cellulose acetate electrophoresis, and ^{125}I-cytochrome *c* staining. Our use of these techniques has been described previously (33).

Characterization of GAGs

Isotopic. The type and the size of GAGs produced by unstimulated and cytokine-stimulated fibroblasts were assessed as described previously (33). In brief, GAGs were isolated by CPC precipitation and sized using Biogel P-10 (Bio-Rad Laboratories, Richmond, CA) and Sepharose CL-2B (Pharmacia LKB Biotechnology Inc., Piscataway NJ) columns. The void volume of the CL-2B column was established using polymerized calf thymus DNA (Sigma Chemical Co., St. Louis, MO). The GAGs were then incubated with protease-free, HA-specific (39) hyaluronidase (*Streptomyces hyaluronyliticus*) (Calbiochem-Behring Corp., La Jolla, CA) (20 U in 0.02 M acetate buffer [pH 5.0] for 16 h at 56°C) and reeluted from the Biogel P-10 column. Since the undigested GAGs appear in the void volume and the digestate appears in the inclusion volume of the column, comparison of the patterns of ^{3}H elution before and after digestion allowed determination of the percentage of label incorporated into HA.

Chemical. The GAGs produced by untreated and cytokine-treated fibroblasts and known GAG controls were applied to individual lanes of cellulose acetate strips, electrophoresed, and stained with ^{125}I-cytochrome *c* as described (33).

Assessment of fibroblast hyaluronate synthetase activity

The cell-free HA synthesizing capacity of untreated and cytokine-treated fibroblasts was assessed by characterizing the ability of fibroblast sonicates to incorporate UDP-[^{14}C]glucuronic acid into CPC-precipitable HA. This was done using the modifications of the techniques of Eppig and Ward-Bailey (40) and Appel et al. (41) we described previously (33).

Characterization of fibroblast HA degradation

We determined whether unstimulated and cytokine-stimulated fibroblasts differed in their ability to degrade [^{3}H]HA.

Preparation of [^{3}H]HA. Confluent fibroblasts in microtiter wells were incubated in complete medium supplemented with 20–30% FBS, and 0.02 ml [^{3}H]glucosamine (150–200 μCi/ml) for 72 h. Pronase digestion and CPC precipitation were used to isolate the HA that was produced. The precipitates were collected by centrifugation, resolubilized in 2 M $MgCl_2$, reprecipitated with alcohol, collected after centrifugation (8,000 *g*, 10 min), resuspended in distilled H_2O and applied to and eluted from a Sepharose CL-2B column using 0.1 M ammonium acetate pH 7. The radiolabeled material that eluted in the void volume of the column was precipitated with alcohol, collected after centrifugation, and resuspended in complete medium. This high molecular weight material was > 98% HA when assessed using the techniques described above and had a specific activity between 0.19 and 0.3 μCi/μg.

Assessment of fibroblast HA degradation. Equal numbers of fibroblasts in microtiter wells were incubated with [^{3}H]HA (5,000 cpm per well), in the presence or absence of cytokines for up to 72 h. At the end of the incubation period pronase was added, the replicates pooled, and the samples applied to and eluted from a Sepharose CL-2B column. The pattern of elution was assessed by scintillation counting. HA digestion caused [^{3}H]HA to move from the void to the included volume of the column.

Demonstration and characterization of fibroblast HAase

Preparation of fibroblast supernatants. Confluent fibroblasts were incubated in complete medium in the presence and absence of cytokines for up to 72 h. At the end of the incubation their supernatants were removed, cleared by centrifugation, and stored at −70°C until usage. The HA degrading activity of these supernatants was assessed without modification (at pH 7.2) or after acidification and/or concentration. Acidification was accomplished in some cases by the direct addition of concentrated formic acid. Concentration was achieved using two techniques. In some experiments, as described by Orkin and Toole (31), saturated ammonium sulfate was added to a final concentration of 95%, carrier BSA was added, the mixture incubated overnight at 4°C, and the pellet saved after centrifugation (65,000 *g* for 1 h). The pellet was then redissolved in and dialyzed against 0.15 M NaCl, 0.1 M sodium formate, pH 3.7, and insoluble material removed by centrifugation. In other experiments, the supernatants were dialyzed against 0.015 M NaCl, 0.01 M sodium formate, pH 3.7, lyophylized, reconstituted in H_2O to 10% of their original volume, and their pH adjusted to

3.7 with formic acid. When required, these techniques were modified to prepare supernatants with pH values between 3.7 and 7, and supernatants with varying concentrations of sodium and formate.

Preparation of fibroblast lysates. Confluent fibroblasts were incubated for up to 72 h in the presence or absence of cytokines, mechanically detached, pelleted, and resuspended in the desired buffer. In most experiments a 0.1 M sodium formate, 0.15 M NaCl, 0.1% Triton X-100 (Sigma) pH 3.7 (TF buffer) was used. To address specific issues, buffers were also prepared which lacked Triton, had pH values between 3.7 and 7.0, or contained varying concentrations of sodium and formate.

Characterization of HA degrading activity. HA degrading capacity was assessed by comparing the patterns of elution of [^{3}H]HA from Sepharose CL-2B or Sepharose CL-6B columns before and after incubation with fibroblast lysates or supernatants. The [^{3}H]HA was prepared as described above. Depending on the experiment, 10^4–10^5 counts of [^{3}H]HA were incubated for up to 16 h in the presence or absence of lysates and/or supernatants. This time period was used in most studies because our preliminary experiments demonstrated that it allowed for maximal HA degradation. The solutions were then applied to and eluted from Sepharose CL-2B or CL-6B columns. These incubations were performed in the presence of 0.5 to 6 mM saccharolactone (Sigma). In specific experiments, incubations were performed after boiling the fibroblast supernatant and/or lysate.

Sodium borotritiide analysis. To determine whether the HA degrading activity in the supernatants and/or lysates was the result of an endohexosaminidase or an endoglucuronidase, sodium borotritiide analysis was performed. Before digestion, 1.4 mg of umbilical cord HA (Sigma) was dissolved in 1 ml of H_2O and 5 ml ammonium hydroxide was added. The solution was immediately transferred into a tube containing 1.8 mg sodium borohydride and incubated on ice for 45 min. Glacial acetic acid was carefully added drop-wise, followed by Dowex 50 H^+. The Dowex 50 H^+ was removed by centrifugation, methanol was added to the supernatant, and the solution evaporated. The evaporation in the presence of acidic methanol was repeated twice. The HA was then redissolved in water and precipitated with 5 vol of sodium acetate-saturated ethanol. A precipitate was allowed to form in the cold overnight and then removed by centrifugation and lyophylized. Recovery was 99–100%. The reduced HA was redissolved in 2 M NaCl and divided into two aliquots. The first aliquot contained 0.2 ml of reduced HA and received 1 ml of acidified fibroblast supernatant. The second tube contained a similar volume of reduced HA and received 1 ml of acidic buffer only. After an overnight incubation at 37°C, concentrated NaOH was added until the pH was greater than or equal to 8, and 5 mCi of NaB^3H_4 was added. The samples were placed on ice for 30 min, acidified with glacial acetic acid and Dowex 50 H^+, evaporated in the presence of acidic methanol, and precipitated with 5 vol of sodium acetate-saturated ethanol as described above. The newly radiolabeled digested HA sample was then applied to and eluted from a Sepharose CL-6B column. The enzymatically digested portion eluting in the included volume of the column was pooled, treated with testicular HAase (Sigma, 700 U, 16 h, 37°C, pH 5.0) and analyzed on a Biogel P-10 column. The testicular HAase-sensitive material appeared in the included volume of this column. It was pooled, hydrolyzed in 1 N HCl (5 h, 100°C), diluted with water and applied to a Dowex 50 H^+ column (bed vol 0.5 ml) and then eluted sequentially with H_2O followed by 0.5 and then 2.0 M HCl. Authentic radiolabeled glucosaminitol was made by reducing glucosamine with borotritiide in a similar fashion. It was also applied to and eluted from the Dowex 50 H^+ column. The run-through obtained with our test and control samples and the radiolabeled glucosaminitol were then diluted with water and applied to a Dowex 1 × 10 column (bed vol 0.5 ml) which was eluted with increasing concentrations of formic acid up to 0.8 M, followed by 2.5 N HCl.

Sizing of end products of digestion. [^{3}H]HA was incubated for 16 h with fibroblast supernatants and/or lysates at pH 3.7 as described above. The products of digestion were then eluted serially from Sepharose CL-2B, Sepharose CL-6B, and Biogel P-10 columns. The Sepharose CL-6B column had a 1 cm × 120 cm bed vol and had been previously calibrated using high molecular weight HA to determine its void volume (Vo), [^{3}H]glucosamine to determine its salt region (V_T), and 30 kD and 10 kD [^{3}H]heparan sulfate (gifts of Dr. Renato Iozzo, Philadelphia, PA) to calibrate its included volume. The end products of digestion of fibroblast HAase were compared to those produced by testicular HAase incubated with HA at its pH optimum. To determine whether the large saccharide end products of digestion produced by the fibroblast supernatants and lysates were the result of a limitation in enzyme quantity, fractions eluting in the included volume of the Sepharose CL-6B column were pooled, redigested with fibroblast-derived HAase, and reanalyzed using these columns. To determine if inhibitors of HAase were present in the fibroblast lysates or supernatants, experiments were performed in which the effect of testicular HAase on [^{3}H]HA degradation was evaluated in the presence and absence of fibroblast lysates (prepared in TF buffer at pH 7.2). The size of the digestates obtained under these control and test conditions were analyzed as noted above.

Characterization of HA-fibroblast binding

Fibroblasts were incubated in the presence and absence of cytokines for up to 72 h, washed, and incubated with [^{3}H]HA in the presence and absence of up to 300 mg/ml of unlabeled HA at 4°C for 4 h. This time point was chosen because our preliminary studies demonstrated that 4 h allowed for maximal binding. At the end of this incubation the monolayers were washed, mechanically detached, and resuspended in PBS. Equal numbers of cells were then pelleted in albumin-treated Eppendorf tubes, resuspended in 0.1% SDS, heated to 100°C, and [^{3}H]HA binding determined by scintillation counting. In these experiments we compared the [^{3}H]HA binding of fibroblasts that had been previously incubated in the presence and absence of streptomyces hyaluronidase to be sure that endogenously produced HA did not obscure surface HA receptors.

Two techniques were used to characterize the specificity of fibroblast-HA binding. In some experiments untreated and cytokine-treated fibroblasts were incubated with [^{3}H]HA in the presence and absence of 300 mg/ml of unlabeled HA, chrondroitin-4-6 sulfate (Sigma), dermatan sulfate (Sigma), or heparin (Sigma), and the ability of these GAG moieties to compete with [^{3}H]HA for cell binding was assessed. In other experiments fibroblasts were incubated with [^{3}H]HA for 4 h. They were then vigorously washed and incubated for 1–2 h in the presence and absence of unlabeled HA, chrondroitin-4-6 sulfate, dermatan sulfate, or heparin (300 mg/ml for each). The ability of these moieties to displace bound HA was then compared.

mRNA isolation and analysis

Total cellular RNA was extracted from fibroblast monolayers, size-fractionated using agarose-formaldehyde gel electrophoresis, transferred to nylon membranes, and hybridized with ^{32}P-labeled cDNA probes, as described previously (42). Clone HCAM short, encoding a portion of CD44, was a gift of Dr. Eugene Butcher (Stanford University, Palo Alto, CA) (43). Clone pCSF-309, a full length cDNA encoding human IL-6, was a gift of Dr. Steven Clark (Genetics Institute, Cambridge, MA). Plasmids containing cDNA encoding the 28 S ribosomal gene were a gift of Dr. S. Adams (University of Pennsylvania, Philadelphia, PA). After hybridization the membranes were washed under high stringency conditions and subjected to autoradiography.

Results

Effects of cytokines on GAG accumulation. Unstimulated fibroblasts produced modest amounts of GAG. As noted previously (33), rIFN-γ and rTNF individually caused a modest increase in fibroblast GAG accumulation and rIFN-γ plus rTNF interacted in a synergistic fashion to further increase GAG accumulation (Table I; Figure 1). These stimulatory effects were seen after as little as 24 h and were more prominent after 72 h of

Table I. Recombinant Cytokine Regulation of Fibroblast [^{3}H]Glucosamine and $Na_2{}^{35}SO_4$ Incorporation

Culture conditions*	[^{3}H]Glucosamine	$Na_2{}^{35}SO_4$
	cpm/well‡	
Control	5,611±40	5,476±694
rIFN-γ	7,639±764	3,813±381
rTNF	9,371±848	4,498±267
rIL-1	11,161±400	3,903±382
rIFN-γ + rTNF	22,030±794	3,208±475
rIL-1 + rTNF	16,564±875	3,261±403

* Fibroblasts were incubated for 72 h in complete medium only (control) or with rIFN-γ (10^3 IU/ml), rTNF (20 ng/ml), and rIL-1-α (2.5 ng/ml) as noted. ‡ Incorporation of $Na_2{}^{35}SO_4$ and [^{3}H]glucosamine as described in Methods.

fibroblast-cytokine incubation (data not shown). In all cases the stimulation was dose dependent with peak effects seen at 10^3 IU/ml of rIFN-γ and 20 ng/ml of rTNF (the highest doses tested). At these concentrations fibroblasts incorporated 145 and 149%, respectively, as much [^{3}H]glucosamine into GAG as controls ($P < 0.01$ vs. control, paired t test). In comparison, fibroblasts incubated with rIFN-γ and rTNF in combination incorporated 428±36% as much [^{3}H]glucosamine into GAG as controls ($P < 0.03$ vs. controls, paired t test). The stimulatory effects of rIFN-γ and rTNF, alone and in combination, were apparent with isotopic and chemical assessment techniques of HA production (Figs. 1 and 2), were negated by neutralizing antibodies against these cytokines (data not shown) and could not be attributed to alterations in cell number (data not shown). In all cases they appeared to be at least partially specific for HA since rIFN-γ and/or rTNF did not have similar stimulatory effects on ^{35}S incorporation into GAG (Table I) or on the production of sulfated GAGs (Fig. 2).

Recombinant IL-1α also stimulated fibroblast GAG production. This effect was dose dependent with peak stimulation noted with 2.5 ng/ml of this cytokine (the highest dose tested) (Fig. 1). At this concentration fibroblasts incubated with rIL-1-α incorporated 190% as much [^{3}H]glucosamine into GAG as controls ($P < 0.001$ vs. control, paired t test). Recombinant IL-1-β had similar stimulatory effects (data not shown). In contrast to the synergistic interaction of rIFN-γ and rTNF, high concentrations of rIL-1 and rTNF interacted, at most, in an additive fashion in stimulating fibroblast GAG production (Figs. 1 and 2). This additive interaction was dose dependent for both cytokines (Fig. 1) and could not be attributed to alterations in cell number (data not shown). The stimulatory effects of rIL-1 and rTNF, alone and in combination, were noted with isotopic and chemical assessments of GAG accumulation (Figs. 1 and 2) and thus could not be explained by changes in [^{3}H]glucosamine pool size. In addition, these stimulatory effects appeared to be at least partially specific for HA since > 95% of the ^{125}I-cytochrome c–binding material produced by these cells was HA (Fig. 2), > 90% of the [^{3}H]glucosamine-labeled material produced by these cells was sensitive to digestion with streptomyces hyaluronidase (data not shown), and these cytokines were unable to significantly stimulate ^{35}S incorporation into sulphur containing moieties (Table I).

Recombinant cytokine regulation of fibroblast hyaluronate synthetase activity. To determine whether cytokine-induced alterations in HA accumulation could be adequately explained by cytokine-induced alterations in HA biosynthesis, we measured the hyaluronate synthetase activity of unstimulated and cytokine-stimulated fibroblasts. Unstimulated fibroblasts manifest modest levels of hyaluronate synthetase activity. In keeping with their effects on HA accumulation, rIFN-γ, rTNF, and rIL-1 individually caused modest increases, and rTNF and rIFN-γ in combination synergistically augmented fibroblast hyaluronate synthetase activity (Table II). Even though rIL-1 and rTNF, in combination, had an additive or lesser effect on HA accumulation, this cytokine combination synergistically augmented hyaluronate synthetase activity. In fact, the levels of hyaluronate synthetase activity in cells stimulated with rIL-1 plus rTNF were consistently greater than the levels seen in cells incubated with rIFN-γ plus rTNF (Table II). Thus, cytokine-induced alterations in hyaluronate synthetase activity do not fully explain the effects of these regulatory agents on fibroblast HA accumulation.

Effect of recombinant cytokines on the size of the HA in fibroblast cultures. We incubated unstimulated and cytokine-stimulated fibroblasts with [^{3}H]glucosamine, isolated the HA that was produced, and obtained an index of its size using Sepharose CL-2B chromatography. As seen in Table III, a cytokine-induced alteration in HA size was detected. This effect was seen after as little as 24 h and was most prominent after 72 h of fibroblast-cytokine incubation. After 72 h of incubation ~ 38% of the HA produced by unstimulated fibroblasts appeared in the void volume of this column. Treatment of fibroblast monolayers with rIFN-γ or rTNF caused a modest increase and treatment with rIL-1 caused a modest decrease in the percentage of HA eluting in the void volume of these columns. Interestingly, > 70% of the HA produced by fibroblasts incubated with rIFN-γ plus rTNF appeared in the Sepharose CL-2B column void-volume effluent. In contrast, only > 21% of the HA produced by fibroblasts incubated with rIL-1 plus rTNF appeared in the Sepharose CL-2B void-volume effluent ($P < .03$ rIFN-γ plus rTNF or rTNF plus rIL-1 vs. control cells, Friedman free rank order analysis) (Table III). These studies demonstrate that rIFN-γ plus rTNF interact in a synergistic fashion to stimulate the accumulation of high molecular weight HA. In contrast, rTNF interacts with rIL-1 to cause an additive increase in HA accumulation with a disproportionate amount of this HA having a low molecular weight.

Recombinant cytokine regulation of HA degradation by intact fibroblasts. The finding that cytokine-induced alterations in fibroblast HA accumulation could not be fully accounted for by cytokine-induced alterations in HA biosynthesis prompted us to determine whether cytokine-induced alterations in HA degradation were involved in this process. In these studies fibroblast HA-degrading capacity was characterized by comparing the patterns of elution from a Sepharose CL-2B column of high molecular weight [^{3}H]HA (radiolabeled HA that previously eluted in the void volume of a Sepharose CL-2B column) before and after incubation with viable cells. Before incubation with fibroblasts > 95% of the [^{3}H]HA eluted in the void volume of the Sepharose CL-2B column. After incubation (72 h, 37°C) with unstimulated fibroblasts ~ 35% of the [^{3}H]HA appeared in the void volume of the column. Recombinant IFN-γ did not significantly alter [^{3}H]HA degradation while recombinant TNF caused a modest decrease and rIL-1 caused

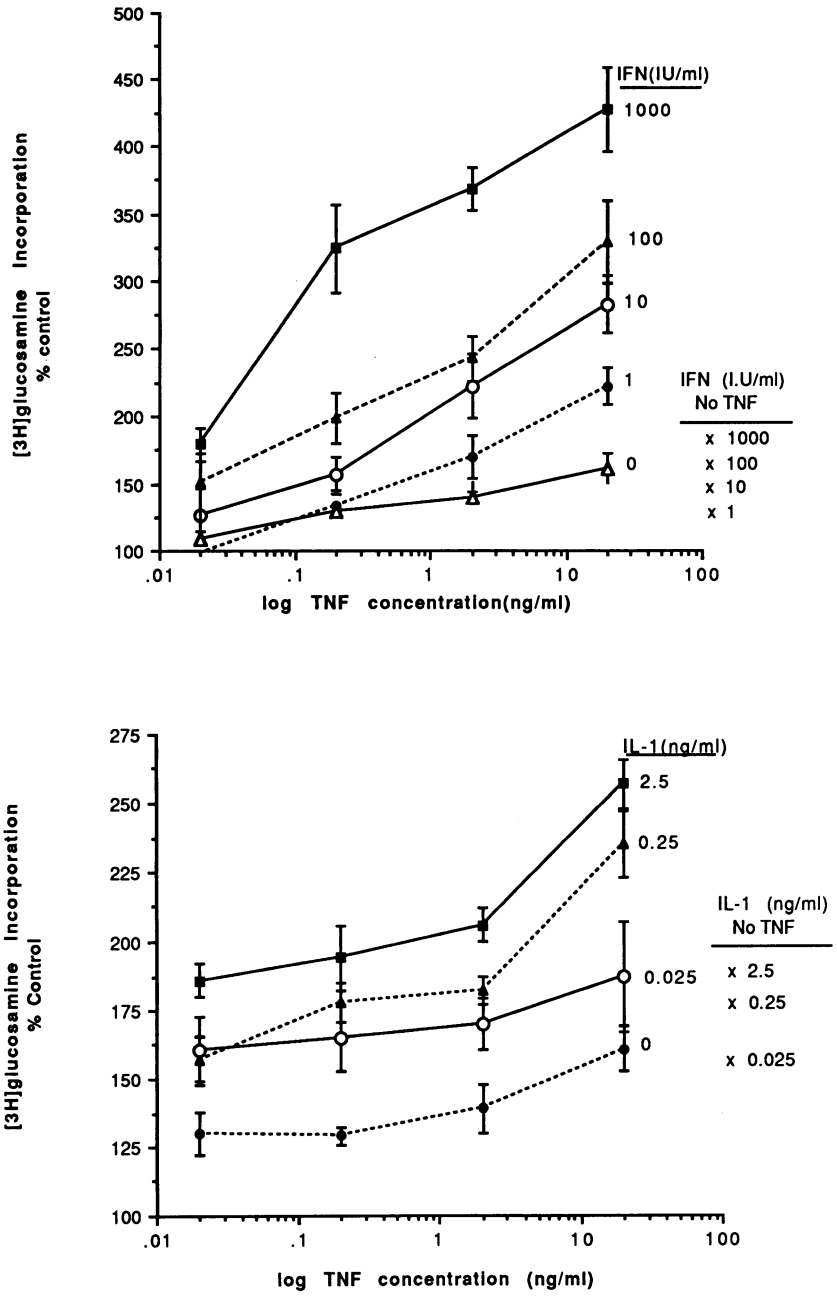

Figure 1. Regulation of lung fibroblast GAG production by recombinant cytokines. In the top panel fibroblasts were incubated for 72 h with [^{3}H]glucosamine in serum-free conditions in the presence of rIFN-γ and/or rTNF. The X on the right side of this panel illustrates the effects of varying doses of rIFN-γ alone. In the bottom panel fibroblasts were similarly incubated with [^{3}H]glucosamine in the presence of rTNF and/or rIL-1-α. The X on the right side of this panel illustrates the effects of varying concentrations of rIL-1 alone. Values represent the mean±SEM of at least 10 experiments. The SEM of the effects of rIFN-γ and rIL-1 individually were $\leq$ 10% of the noted values.

a modest increase in HA degradation (Table III). Incubating fibroblasts with rIFN-γ plus rTNF caused a marked decrease in fibroblast HA degradation, with ~ 67% of the [^{3}H]HA incubated with these cells appearing in the void volume of the column. In contrast, rIL-1 plus rTNF increased fibroblast HA degradation with only ~ 18% of the [^{3}H]HA incubated with these cells eluting in the Sepharose CL-2B void volume ($P < .05$ rIFN-γ plus rTNF and rIL-1 plus rTNF vs. control cells, Friedman free rank order analysis) (Table III). These regulatory effects could be seen after as little as 24 h and were most prominent after 72 h of [^{3}H]HA-fibroblast-cytokine incubation (data not shown). In addition, these effects could not be explained by fibroblast-derived unlabeled HA competing with [^{3}H]HA for degradation since the amount of HA added to these fibroblast monolayers was 15–40 times greater than the maximum amount of HA produced by unstimulated or cytokine-stimulated fibroblasts. When these observations are viewed with the previous data they demonstrate that the synergistic increase in the production of high molecular weight HA caused by rIFN-γ plus rTNF is, at least partly, the result of the ability of these cytokines to synergistically stimulate fibroblast hyaluronate synthetase activity while simultaneously inhibiting fibroblast HA degradation. In contrast, rIL-1 plus rTNF synergistically stimulate fibroblast hyaluronate synthetase activity while simultaneously stimulating fibroblast HA degradation. This explains, at least in part, why cells incubated with these cytokines accumulate less total HA and a smaller HA than cells incubated with rIFN-γ plus rTNF.

Demonstration and preliminary characterization of fibroblast-derived HAase. A number of groups have reported that normal and abnormal human skin fibroblasts lack HAases (31, 32). The HAase content of other normal human fibroblasts

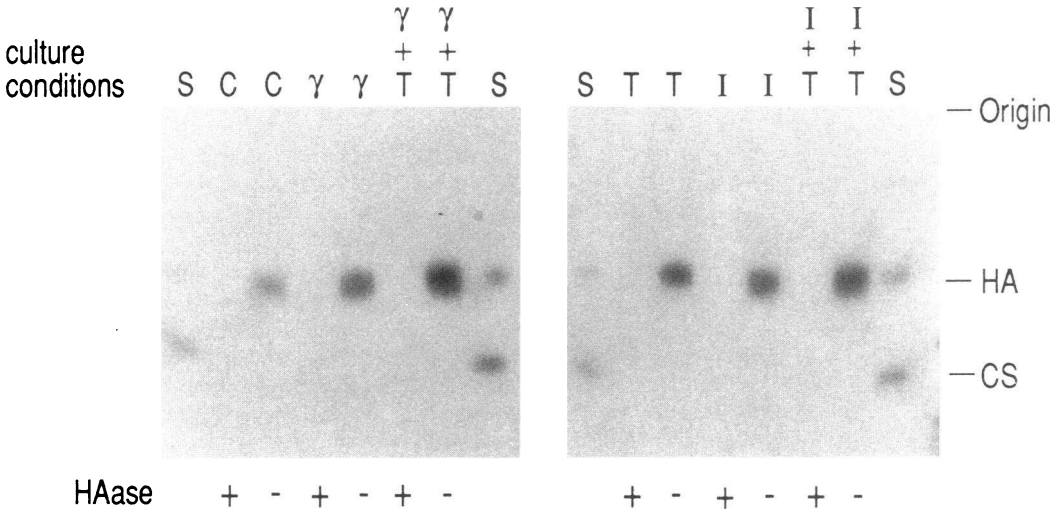

Figure 2. Chemical analysis of fibroblast GAG production. Fibroblasts were incubated with rIFN-γ (γ) (10^3 IU/ml), rTNF (*T*) (20 ng/ml), and rIL-1-α (*I*) (2.5 ng/ml), alone and in combination, as noted. GAG production was assessed using cellulose acetate electrophoresis, ^{125}I-cytochrome *c* staining and autoradiography as described. GAG analysis was performed on samples that had been incubated with (+) and not exposed to (−) active streptomyces hyaluronidase. Authentic HA and chondroitin 6 sulphate (*CS*) were run in lanes containing standards (*S*).

has not been assessed. Thus, to characterize the mechanism by which cytokines regulate fibroblast HA degradation, we first had to determine whether human lung fibroblasts produce or contain HA degrading enzymes. To accomplish this, we compared the pattern of elution of high molecular weight [^{3}H]HA from a Sepharose CL-2B column before and after incubation with fibroblast supernatants and cellular extracts. At pH 7.2 neither the supernatants nor the extracts contained HA degrading activity (Fig. 3 and data not shown). However, both contained significant amounts of HA degrading activity when prepared under specific conditions. Cell-associated HA degrading activity was noticed only when detergent lysis was employed and the lysates acidified to the pH 3.7–4.0 range (Fig. 3). Supernatant HA degrading activity was maximal at a pH of 3.7–4.0 (Fig. 4). In all cases the HA degrading activity was abrogated by boiling and negated in the presence of 3 M NaCl (data not shown).

To size the products of digestion of this fibroblast-derived HA degrading activity, high molecular weight [^{3}H]HA was incubated with the fibroblast enzyme at pH 3.7 and the products of digestion isolated and sized using calibrated Sepharose CL-6B and Biogel P-10 columns. These studies demonstrated that the major digestion products of this fibroblast-derived enzyme were 10–40-kD molecular mass saccharides that eluted in the inclusion volume of the Sepharose CL-6B column (K_{av} = 0.3–0.4) and the void volume of the Biogel P-10 column. In contrast, testicular HAase produced saccharides with a K_{av} = 0.81 on the Sepharose CL-6B column that eluted in the inclusion volume of the Biogel P-10 column (Fig. 5 and data not shown). Exposure of the 10–40-kD saccharides produced by the fibroblast enzyme to additional fibroblast enzyme did not cause significant further degradation. The products of the second digestion still eluted in the inclusion volume of the Sepharose CL-6B column and the void volume of the Biogel P10 column (data not shown). This suggests that the large saccharide end products of digestion of the fibroblast-derived HA degrading enzyme are not produced as a result of incomplete hydrolysis due to limitations in the amount of enzyme (30). In addition, this large saccharide did not appear to be produced as a result of an inhibitor of testicular type hyaluronidases since fibroblast supernatants and detergent lysates at pH 7.2 did not significantly inhibit the degradative capacity of testicular HAase (data not shown).

The fibroblast-derived HA degrading activity appeared to be an endoglycosidase and not an exoglycosidase since saccharolactone, a known inhibitor of β-glucuronidase, was included in our digestions. In addition, in the absence of saccharolactone, digestions with the fibroblast-derived enzyme produced predominantly 10–40-kD saccharides even though β-glucuronidase plus β-*N*-acetylhexosaminidase produced monosaccharides when incubated with the same substrate (Fig. 5 and data not shown). To determine if this endoglycosidase was an endohexosaminidase or an endoglucuronidase, sodium borotritiide analysis was employed. In these experiments ~ 70% of the radiolabeled material in the fibroblast digestate adhered to the cation exchange column but none bound to the anion exchange column (data not shown). Authentic glucosaminitol behaved similarly, with 80% retained on the cation exchange column and none binding to the anion exchange column. Fur-

Table II. Recombinant Cytokine Regulation of Fibroblast HA Synthetase Activity

		Fibroblast culture conditions*				
Experiment	Control	IFN-γ	TNF	IL-1	IFN-γ + TNF	IL-1 + TNF
1	464.4‡	573.9	921.5	647.3	1,689.9	2,850.5
2	828.1	1,076.3	1,251.0	916.5	2,011.6	4,591.5

* Fibroblasts were incubated for 72 h in complete media only (control) or with rIFN-γ (10^3 IU/ml), rTNF (20 ng/ml), and/or rIL-1-α (2.5 ng/ml) as noted. ‡ HA synthetase activity per 2.5 × 10^5 cells assessed as described in Methods. Values represent means of duplicate determinations that were within 10% of each other.

Table III. Recombinant Cytokine Regulation of the Size of Fibroblast-derived HA, Fibroblast HA Degradation, Fibroblast HAase Activity, and Fibroblast HA Binding

Fibroblast culture conditions*	HA Size‡	HA Degradation§	HAase Activity % control‖	HA binding (CPM/10^6 cells)¶
Control	38.2±9	34.8±4.9	—	370.6±35
IFN-γ	43.6±4.2	32.8±6.3	102.7±13.1	489.6±57
TNF	53.2±7	44.4±5.1	158.9±22.3	388.0±43
IL-1	34.3±1.9	23.9±3.1	174.9±29.5	481.2±91
IFN-γ + TNF	71.8±6.5	66.5±7.3	130.8±9.1	80.5±9
IL-1 + TNF	21.3±2.1	17.2±1.2	110.9±6.7	722.0±87

* Fibroblasts were incubated in complete medium only (control) or with rIFN-γ (10^3 IU/ml), rTNF (20 ng/ml), and/or rIL-1-α (2.5 ng/ml) as noted. ‡ Fibroblast-cytokine incubation was performed in the presence of [^{3}H]glucosamine. At the end of the incubation period the pronase resistant CPC-precipitable material was applied to and eluted from a Sepharose CL-2B column. Values represent the mean±SEM of percentage of total counts eluting in the void volume of the column in ≥ four experiments. § Fibroblast-cytokine incubation was performed in the presence of high molecular weight [^{3}H]HA. At the end of the incubation the pronase-resistant CPC-precipitable material in the wells was collected and eluted from a Sepharose CL-2B column. The percentage of the [^{3}H]HA eluting in the void volume of the column was determined as described in Methods. Greater than 95% of the [^{3}H]HA appeared in the void volume when similarly incubated in medium without cells. Values represent the mean±SEM ≥ five experiments. ‖ Lysates were prepared in TF buffer pH 3.7 and incubated with high molecular weight [^{3}H]HA as described in Methods. The HA degrading activity of lysates from cytokine-stimulated cells is expressed as a percentage of that in cells incubated under control conditions. Values represent mean±SEM of ≥ nine experiments. ¶ Unstimulated and cytokine-stimulated fibroblasts were incubated for 4 h at 4°C with [^{3}H]HA and specific HA binding determined as described in Methods. Values represent the mean SEM of ≥ four experiments.

thermore, the radiolabeled end products of digestion and glucosaminitol had similar patterns of elution from the Dowex 50 column. This demonstrates that the fibroblast enzyme leaves only *N*-acetylglucosamine as the reducing sugar, and thus is an endohexosaminidase; i.e., a hyaluronidase. When viewed in combination these studies demonstrate that lung fibroblasts produce a cell-associated and soluble HAase(s), which functions best at low pH and whose end products of digestion are larger than those of testicular HAase.

Recombinant cytokine regulation of fibroblast HAase activity. Detergent lysates (pH 3.7) were prepared from fibroblasts that had been incubated for 24–72 h in the presence and absence of cytokines. The HAase activity of these lysates was evaluated by comparing the elution of high molecular weight [^{3}H]HA from a Sepharose CL-2B column before and after lysate exposure. Greater than 95% of the [^{3}H]HA appeared in the void volume of the Sepharose CL-2B column before exposure to cell lysates. After incubation with lysates from unstimulated fibroblasts ~ 37% of the [^{3}H]HA eluted in the void volume of the column (Table III). Recombinant IFN-γ did not alter, while rTNF and rIL-1 caused modest increases in HAase activity. Recombinant IFN-γ plus rTNF and rIL-1 plus rTNF also caused modest increases in HAase activity (Table III). However, the former cytokine combination inhibited while the latter combination augmented HA degradation. Thus, although detergent lysates of human lung fibroblasts contain HAase activity, the levels of this activity did not correlate fully with the HA degrading capacity of unstimulated and cytokine stimulated fibroblasts.

Cytokine regulation of HA-fibroblast binding. The pH profile of fibroblast HAase suggests that it is a lysosomal enzyme whose function requires HA binding and internalization (3, 27, 28). Since the HA degrading activity of whole cells did not correlate with that of cell lysates, we speculated that cellular HA degradation was regulated at the level of cellular HA binding. To test this hypothesis we compared the ability of unstimulated and cytokine-stimulated fibroblasts to specifically bind HA. Unstimulated fibroblasts bound modest amounts of HA. Recombinant IFN-γ, rTNF, and rIL-1 caused modest increases in this binding. Recombinant IFN-γ plus rTNF, a cytokine combination that inhibited cellular HA degradation, caused a significant decrease in HA binding (Table III). In contrast, rIL-1 plus rTNF, a cytokine combination that increased cellular HA degradation, increased HA binding (Fig. 3). These cytokine-induced alterations in HA binding were seen after as little as 24 h of cytokine-fibroblast incubation. In addition, they could not be attributed to effects of endogenously produced HA since binding was not altered by preincubation with streptomyces HAase (data not shown). In all cases, the receptor(s) involved in this binding was at least partially specific for HA since concentrations of chrondroitin sulfate, dermatan sulfate and heparin as high as 300 mg/ml competed weakly, if at all, with HA for cellular binding (data not shown). These studies demonstrate a correlation between cytokine-induced alterations in the HA degrading activity and cellular HA binding.

Cytokine regulation of CD44 mRNA. Studies were undertaken to determine if cytokine-induced alterations in HA-fibroblast binding were associated with comparable alterations in the levels of mRNA encoding CD44, a major cellular HA receptor (43, 44). Unstimulated normal lung fibroblasts expressed three mRNA isoforms encoding CD44. Recombinant IL-1 and rTNF, individually, augmented all three CD44 isoforms with rIL-1 being more potent (Fig. 6). Incubating fibroblasts with rIL-1 and rTNF in combination caused a further increase in CD44 mRNA accumulation (Fig. 6). This effect was seen after as little as 4 h of fibroblast-cytokine incubation (data not shown). In contrast, rIFN-γ did not significantly alter, or decreased the levels of CD44 mRNA in unstimulated and rTNF stimulated cells (Fig. 6). When these blots were probed for IL-6 mRNA a distinctly different pattern of hybridization was noted (Fig. 6). The levels of steady state mRNA for

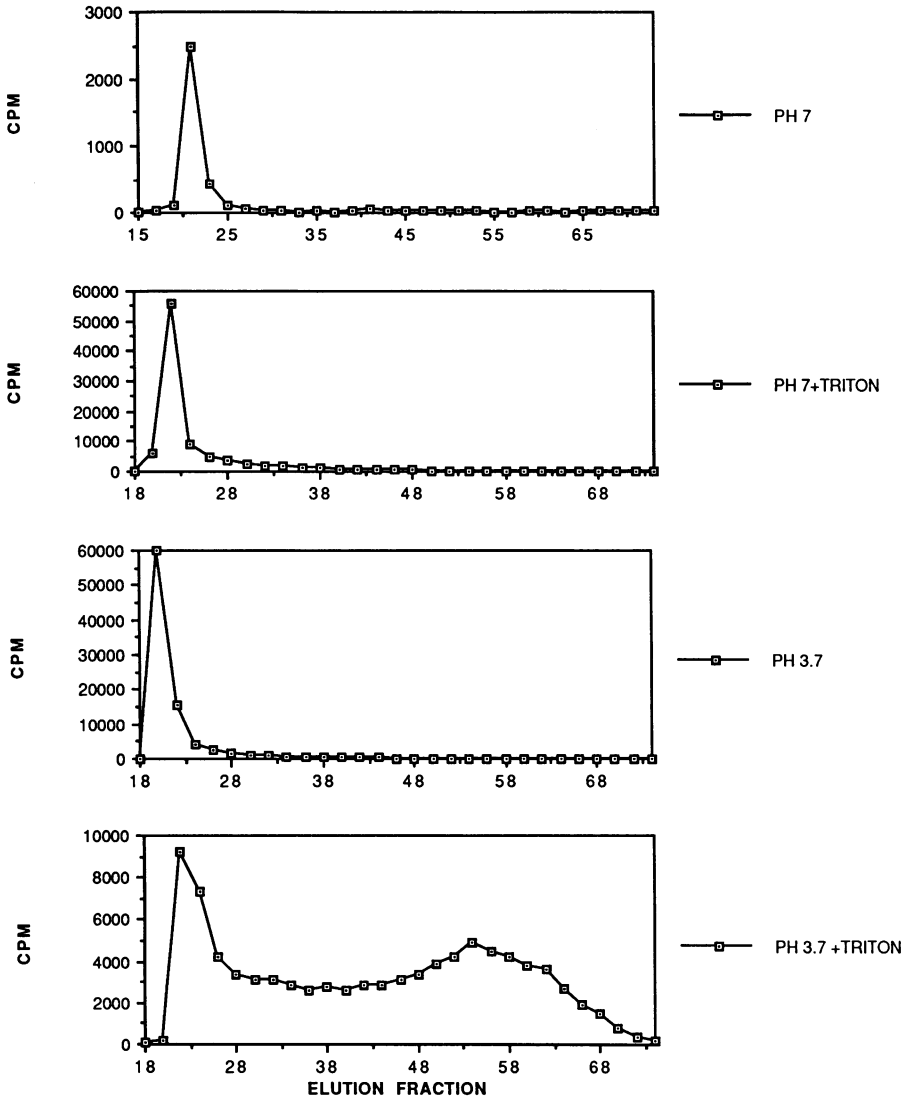

Figure 3. Demonstration of fibroblast HAase activity. Fibroblast extracts were prepared at pH 7 and pH 3.7 in the presence or absence of detergent as described in Methods. The elution pattern of high molecular weight [^{3}H]HA from a Sepharose CL-2B column after incubation with these extracts was determined. HA degradation caused the radiolabeled HA to move into the included volume (tubes 24–70) of the column. Greater than 95% of the high molecular weight [^{3}H]HA eluted in the void volume of the column when incubated in complete medium only.

28 S were not similarly altered by these cytokines (Fig. 6). Thus, only cytokines and cytokine combinations that increase HA binding and cellular HA degradation (rIL-1, rIL-1 plus rTNF) significantly augment CD44 mRNA levels. This suggests that the alterations in HA degradation and HA binding induced by rIL-1 and rTNF may be, at least partially, mediated by the ability of these agents to regulate the expression of CD44.

Discussion

To further understand the processes regulating HA accumulation in normal and diseased tissues we analyzed the effects of rTNF, rIFN-γ, and rIL-1, alone and in combination, on fibroblast HA production. Our studies demonstrate that these cytokines selectively stimulate fibroblast HA production and that rTNF interacts in a synergistic fashion with rIFN-γ and in an additive fashion with rIL-1 to further augment the production of high and low molecular weight HA, respectively. They also demonstrate that the synergistic interaction of rTNF and rIFN-γ is the result of the ability of these cytokines to synergistically augment HA synthesis while simultaneously decreasing fibroblast HA degradation. In contrast, the effects of rIL-1 and rTNF in combination were shown to result from a synergistic increase in HA synthesis and a simultaneous increase in HA degradation. We have also demonstrated that human lung fibroblast HA degradation is mediated, at least in part, by a previously unappreciated lysosomal-type HAase and that these cytokines likely regulate lung fibroblast HA degradation by altering fibroblast-HA binding rather than the levels of fibroblast HAase activity. Lastly, these studies demonstrate that cytokine-induced alterations in HA degradation and HA-fibroblast binding are mirrored by alterations in CD44 mRNA accumulation. The demonstration that these cytokines regulate fibroblast HA production is in accord with prior observations from this and other laboratories (33, 34). The present studies expand on these earlier observations since they show, for the first time, that these important cytokines interact to alter the size as well as the amount of HA produced by fibroblasts, that these alterations are mediated, at least in part, by alterations in fibroblast HA degradation, that human fibroblasts contain a lysosomal-type HAase and that fibroblast HA degradation may be regulated by alterations in fibroblast-HA binding rather than

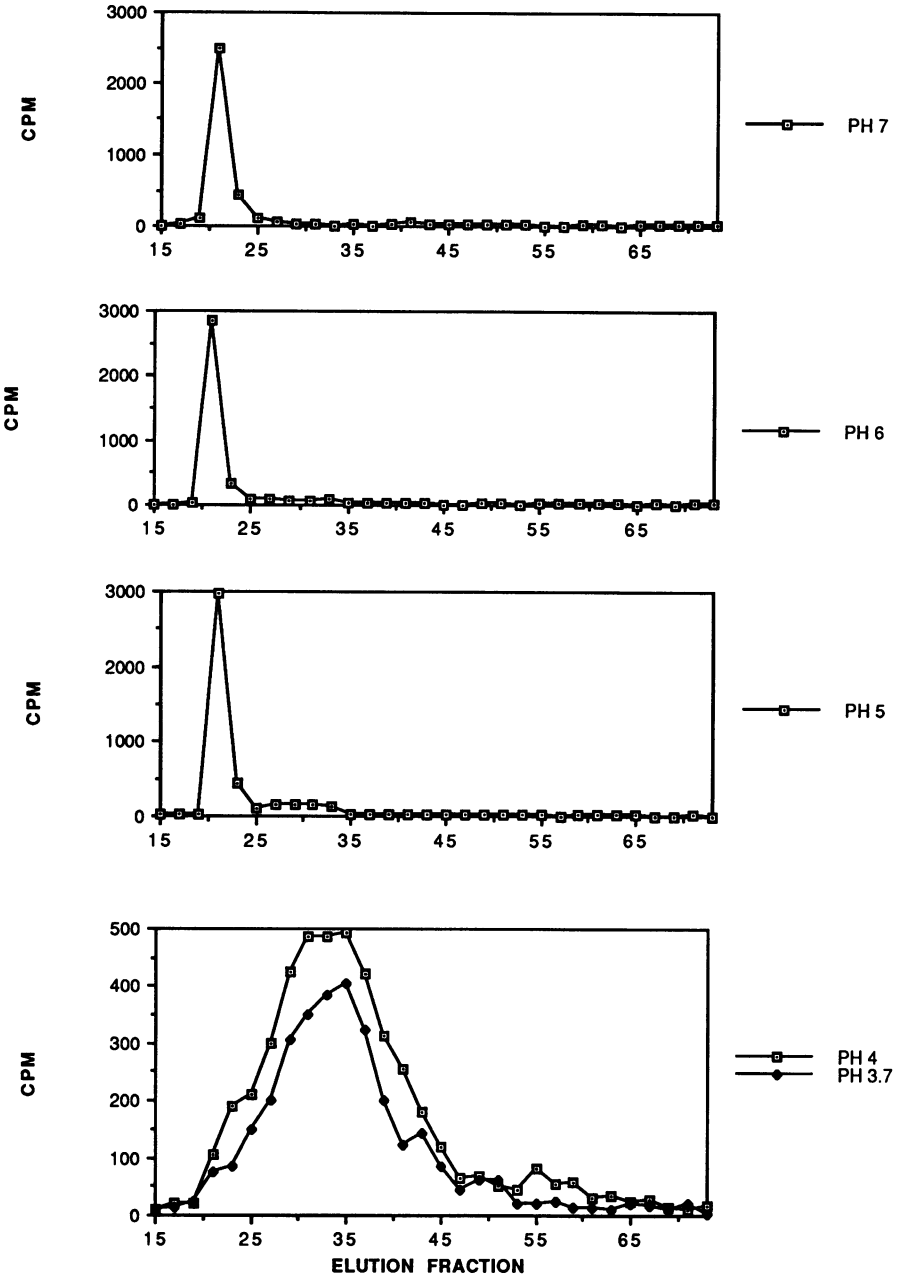

Figure 4. Demonstration of the pH profile of fibroblast-derived HAase. Fibroblast HAase was prepared in TF buffer at the noted pH values and its ability to degrade [^{3}H]HA characterized as described in Methods. Greater than 95% of the [^{3}H]HA used in these experiments eluted in the void volume of the column (tubes 18–24) when incubated in complete medium only.

the levels of fibroblast HAase activity. They are also the first studies to suggest that CD44 is involved in this regulatory process.

Lysosomal HAases are part of an intracellular apparatus designed to degrade HA and chondroitin sulphates in vivo. This apparatus requires HA internalization, usually via HA receptors, followed by the concerted action of HAase, β-D-glucuronidase and β-*N*-acetyl-D-hexosaminidase (27–31). Despite the importance of HAase in this degradative pathway, this enzyme is not present in all cell types. Specifically, a number of investigators have reported that, in contrast to nonhuman fibroblasts (12, 28, 31) and human rheumatoid synovial cells (31), normal human fibroblasts lack HAase (31, 32). However, our studies demonstrate that normal human lung fibroblasts produce an endohexosaminidase which functions best at pH 3.7. By all parameters this enzyme is a lysosomal HAase. Like other lysosomal HAases (31, 45), cell-associated and soluble forms of this enzyme have been noted. An interesting aspect of these studies is the demonstration that this fibroblast-derived HAase produces HA end products of digestion that are surprisingly large. Exhaustive digestion of HA with testicular HAase results in a mixture of oligosaccharides consisting mainly of tetrasaccharides with a substantial portion of hexasaccharides and smaller amounts of octasaccharides and disaccharides (27). In contrast, digestion with fibroblast HAase results only in 10–40-kD saccharides. This observation may explain, at least in part, why previous investigators were unable to detect this enzymatic activity. Other items that may have clouded prior appreciation of this enzyme include the use of acetate buffers which inhibit the function of some lysosomal HAases and a lack of detergent extraction (31, 32, 46). Alternatively, it is possible that the contrast between our findings with lung fibroblasts and previous results with skin fibroblasts represents a true tissue specific difference. Support for this possibility comes from stud-

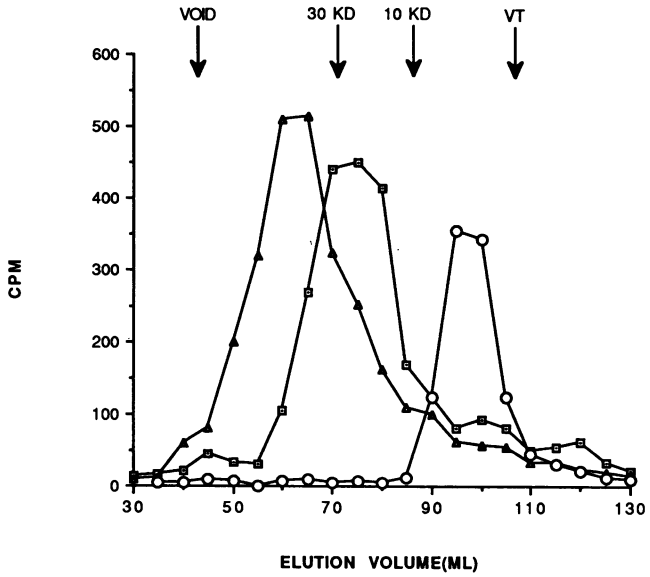

Figure 5. Demonstration of the size of the end products of digestion of fibroblast HAase. High molecular weight [^{3}H]HA was incubated with the HAase in the supernatants (supnt) and detergent lysates (lysate) of lung fibroblasts at pH 3.7. The same substrate was also incubated with testicular HAase at pH 5.0. The products of digestion were then isolated and sized using a calibrated CL-6B column as described in the methods. The void volume and salt region (*VT*) are illustrated. ⊡, supnt; △, lysate; ○, testic HAase.

ies demonstrating that TGF-β differentially regulates human lung and skin fibroblast HA production (35) and preliminary studies in our laboratory which failed to demonstrate significant amounts of HAase activity in CRL1445 human skin fibroblasts (obtained from the American Type Culture Collection) (Sampson, P., and J. A. Elias, unpublished observation).

Cytokine-induced alterations in HA size could result from alterations in HA degradation and/or alterations in the size of the HA molecules that are produced. Although we cannot absolutely rule out this latter possibility, the demonstration that cytokine-stimulated fibroblasts that produce low molecular weight HA have a heightened ability to degrade exogenous HA while cells that produce high molecular weight HA have a depressed ability to degrade exogenous HA suggests that the effects that have been noted are, at least partially, the result of alterations in cellular HA degradation. Importantly, cytokine-induced alterations in fibroblast HA degradation could not be explained by alterations in fibroblast HAase activity but were paralleled by alterations in HA-fibroblast binding and the levels of cellular CD44 mRNA. This suggests that cytokines may regulate fibroblast HA degradation by altering fibroblast-HA binding and that CD44, a recently cloned HA receptor (43, 44), may play a role in this process. The polymorphic nature of CD44 (47) and the complicated nature of the large number of other HA-binding structures (48–50) mandate further study before the HA-binding structure(s) involved in this process can be definitely identified. Additional investigation is also required to determine if our findings in cells cultured on plastic are applicable to cells growing on other matrices.

Our studies demonstrate for the first time that IL-1 and TNF, in combination, cause human lung fibroblasts to produce low molecular weight HA. This observation is in accord with previous studies demonstrating that connective tissue activating peptide (37) and migration stimulating factor (51) alter the quantity and size of the HA produced by fibroblasts. On a per weight basis, low molecular weight HA has a greater oncotic effect than high molecular weight HA. Thus, fibroblast production of low molecular weight HA and fibroblast degradation of high molecular weight HA augments interstitial fluid retention and may contribute to the pathogenesis of the edema common to all injuries. The oncotic effects of low molecular weight HA may be particularly important in the pathogenesis of adult respiratory distress syndrome (52) and renal transplant rejection (53) where HA abnormalities and tissue edema are prominent findings.

The findings in this study have important implications for the pathogenesis of the HA abnormalities seen in a variety of inflammatory disorders. When compared to normals, patients with sarcoidosis have lung mononuclear cells that spontaneously elaborate IFN-γ (54), TNF (55), and IL-1 (56), and

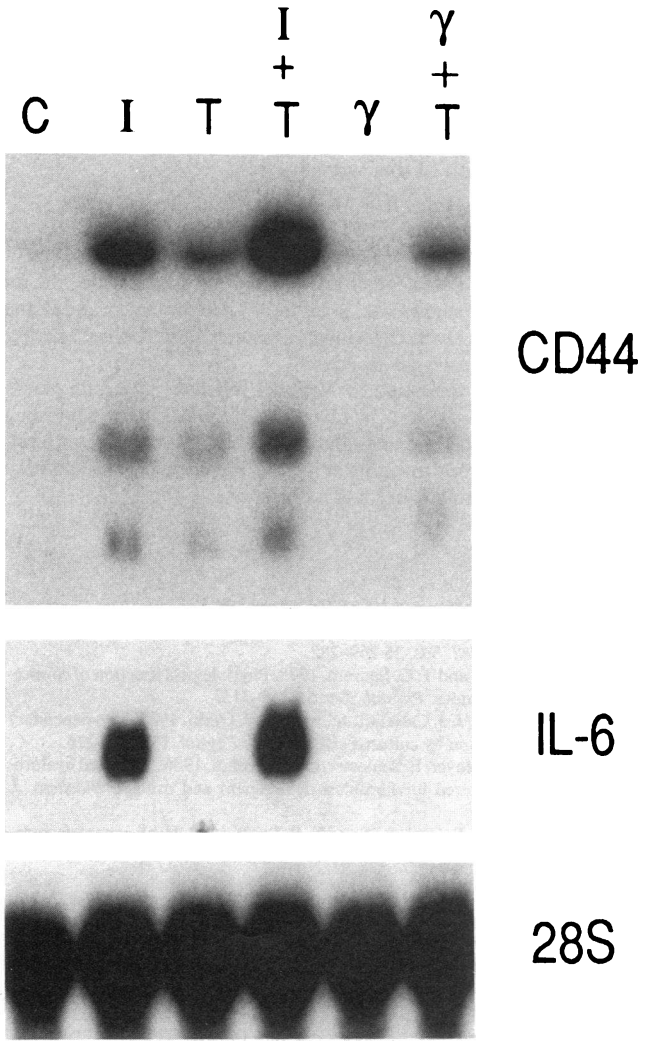

Figure 6. Steady-state CD44, IL-6, and 28S mRNA levels. Fibroblasts were incubated for 16 h in complete medium alone (control) (*C*) or with rIL-1-α (2.5 ng/ml) (*I*), rTNF (20 ng/ml) (*T*), and rIFN-γ (10^3 IU/ml) (γ), alone and in combination as noted. Total cellular RNA was extracted and CD44 (*top panel*), IL-6 (*middle panel*), and 28S (*bottom panel*) mRNA were assessed.

increased levels of HA in their bronchoalveolar lavage fluid (22). Patients with scleroderma have excessive amounts of HA in their skin (16), increased serum HA levels that correlate with disease activity (15) and dermal fibroblasts that elaborate abnormally large amounts of HA in vitro (57). Similarly, the elevated levels of HA in the lavage fluid and tissues of animals with bleomycin-induced lung disease and patients with idiopathic pulmonary fibrosis, delayed type hypersensitivity reactions, asthma, and psoriasis, are associated with chronic tissue inflammation characterized by activated mononuclear cells (20, 23, 26, 58). Rheumatoid arthritis is a particularly interesting disease with regard to HA metabolism. Patients with rheumatoid arthritis have elevated levels of HA in their serum (17) and joint space (17) and synovial tissues that are infiltrated with activated macrophages and lymphocytes that produce exaggerated amounts of IL-1 and TNF (59, 60). The HA found in the joints of these patients has a very low viscosity and molecular weight ($\sim$ 50,000) (18). In addition, freshly isolated and propagated rheumatoid synovial fibroblasts produce abnormally depolymerized HA (18). When viewed in combination, these observations suggest that dysregulated production of IL-1, TNF and/or IFN-γ is, at least partly, responsible for the abnormal HA accumulation noted in these disorders. In rheumatoid arthritis it is tempting to speculate that the effects of IL-1 and TNF predominate contributing to rheumatoid synovial cell production of low viscosity HA.

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