Regulation of Human Lung Fibroblast Glycosaminoglycan Production by Recombinant Interferons, Tumor Necrosis Factor, and Lymphotoxin

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Abstract

Mononuclear cells may be important regulators of fibroblast glycosaminoglycan (GAG) biosynthesis. However, the soluble factors mediating these effects, the importance of intercytokine interactions in this regulation and the mechanisms of these alterations remain poorly understood. We analyzed the effect of recombinant (r) tumor necrosis factor (TNF), lymphotoxin (LT), and γ, α, and β_1 interferons (INF-γ, -α and -β_1), alone and in combination, on GAG production by normal human lung fibroblasts. rTNF, rLT, and rINF-γ each stimulated fibroblast GAG production. In addition, rIFN-γ synergized with rTNF and rLT to further augment GAG biosynthesis. In contrast, IFN-α_A, -α_D, and -β_1 neither stimulated fibroblast GAG production nor interacted with rTNF or rLT to regulate GAG biosynthesis. The effects of the stimulatory cytokines and cytokine combinations were dose dependent and were abrogated by the respective monoclonal antibodies. In addition, these cytokines did not cause an alteration in the distribution of GAG between the fibroblast cell layer and supernatant. However, the stimulation was at least partially specific for particular GAG moieties with hyaluronic acid biosynthesis being markedly augmented without a comparable increase in the production of sulfated GAGs. Fibroblast prostaglandin production did not mediate these alterations since indomethacin did not decrease the stimulatory effects of the cytokines. In contrast, protein and mRNA synthesis appeared to play a role since the stimulatory effects of the cytokines were abrogated by cyclohexamide and actinomycin D, respectively. In addition, the cytokines and cytokine combinations increased cellular hyaluronate synthetase activity in proportion to their effects on hyaluronic acid suggesting that induction of this enzyme(s) is important in this stimulatory process. These studies demonstrate that IFN-γ, TNF, and LT are important stimulators of fibroblast GAG biosynthesis, that interactions between these cytokines may be important in this regulatory process, that these cytokines predominantly stimulate hyaluronic acid production and that this effect may be mediated by stimulation of fibroblast hyaluronate synthetase activity.

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Introduction

Glycosaminoglycans (GAGs)[1] are important components of the extracellular matrix. They provide cellular support, regulate cellular biosynthesis (1), proliferation (2), and differentiation (3), and direct the movements of interstitial fluid and macromolecules (4–6). A low level of GAG production is noted in the normal matrix. Increased GAG production is associated with injury (5) and excessive GAG accumulation is characteristic of fibrotic disorders (5, 7–9). Changes in the relative distribution of the different GAG moieties are also seen during tissue morphogenesis (10, 11) and fibrosis (12). Fibroblasts are major producers of GAGs and abnormal fibroblast GAG production may play a role in the pathogenesis of scleroderma (13) and rheumatoid arthritis (14). However, the processes that control fibroblast GAG biosynthesis are poorly understood.

Mononuclear cells are important regulators of fibroblast proliferation and collagen biosynthesis. A mononuclear cell predominant inflammatory cell infiltrate is also noted after many injuries and in many fibrotic disorders. This has led to the speculation that mononuclear cells may also be important regulators of GAG biosynthesis. Support for this concept comes from studies demonstrating that supernatants from activated mononuclear cells stimulate fibroblast GAG biosynthesis (9, 15). However, the factors responsible for these stimulatory effects and the mechanisms of their effect have not been fully characterized.

Activated mononuclear cells elaborate interferons (IFN) (16), tumor necrosis factor (TNF), and lymphotoxin (LT) (17). Gamma interferon (IFN-γ) and beta inteferon (IFN-β) regulate fibroblast growth (16, 18) and collagen biosynthesis (16, 19). TNF and LT have a wide spectrum of cytoregulatory effects (20–26),[2] including an ability to modulate fibroblast proliferation (20)[2] and prostaglandin production (21, 23). In addition, IFN-γ interacts synergistically with TNF and LT in modulating a number of cellular activities (20–22, 27).[2] These observations have led to the speculation that TNF, LT, and IFN might be important regulators of the quantity and types of GAGs produced by normal fibroblasts. We also postulated that interactions between IFN, TNF, and LT might be important in this regulatory process. To test these hypotheses, we characterized the effect of recombinant IFN (γ, α, and β), TNF and LT, alone and in combination, on GAG production by normal lung fibroblasts. Studies were also undertaken to de-

1. *Abbreviations used in this paper:* CPC, cetylpyridinium chloride; GAG, glycosaminoglycan; LT, lymphotoxin; TNF, tumor necrosis factor.
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termine whether these cytokines altered a specific GAG moiety and the mechanism of the effect.

Methods

Cytokines and antibodies

Human recombinant (r) IFN-γ (1.4×10^8 IU/mg protein), IFN-α_A (3×10^6 IU/mg protein), and IFN-α_D (5×10^8 IU/mg protein) were kindly supplied by Hoffmann-LaRoche Inc. (Nutley, NJ). Human rIFN-β_1 (180×10^8 IU/mg protein) was obtained from Triton Biosciences Inc. (Alameda, CA). rTNF (5×10^7 U/mg protein) and rLT (22.1×10^7 U/mg protein) were kindly supplied by Dr. H. Michael Sheppard (Genentech Inc., San Francisco, CA). The cytokines were added directly to fibroblast cultures after dilution. Monoclonal antibody to IFN-γ was obtained from Meloy Laboratories (Springfield, VA) and courtesy of Dr. H. Michael Sheppard (Genentech, Inc.). Monoclonal anti-TNF and anti-LT antibodies were also obtained from Genentech, Inc.

Fibroblast cell lines

Five adult human lung fibroblast lines were used in these studies. Two lines were prepared by the authors from histologically normal areas of lungs resected for therapeutic reasons. The preparation and proliferative characteristics of these lines have been described (28, 29). Normal adult lung fibroblast cell lines (CCL-202, CCL-210, and CCL-200) were also obtained from the American Type Culture Collection (Rockville, MD). All fibroblasts were grown to confluence in Dulbecco's Modified Eagle Medium supplemented with nonessential amino acids (Gibco, Grand Island, NY), penicillin, streptomycin (complete medium) and 10% heat-inactivated FBS (from Gibco). All cell lines were free of mycoplasma contamination and responded similarly to the cytokines being assessed.

Assessment of fibroblast GAG biosynthesis

Isotopic. Fibroblast GAG production was assessed using a modification of previously described methods (9). Fibroblasts were harvested after exposure to trypsin and plated in 96-well flat-bottom microtiter plates (Falcon Labware, Becton Dickinson Co., Oxnard, CA) at 10×10^3/well. Cells were grown to confluence in complete medium supplemented with 10% FBS. The spent medium was then replaced with complete medium with or without cytokine(s) and isotope was added. In most experiments, [^{3}H]glucosamine (10 μl, 8 μCi/ml; New England Nuclear, Boston, MA) was used. When specifically assessing the production of sulfated GAG, $Na_2{}^{35}SO_4$ (10 μl, 20 μCi/ml; ICN Radiochemicals, Irvine, CA) was added. In some experiments, ascorbic acid (50 μg/ml final concentration) was also added. Experiments were performed serum free or with a final well concentration of 10% FBS. All conditions were assessed in triplicate or quadruplicate. The fibroblasts were incubated for up to 72 h at 37°C in 5% CO_2 and air before the cells and their supernatant were digested with pronase (10 μl, 70 mg/ml; Calbiochem-Behring Co., San Diego, CA) for 4 h. Carrier hyaluronic acid was then added (0.1 ml, 2.8 mg/ml; Sigma Chemical Co., St. Louis, MO), and the GAG precipitated with 0.04 ml of 5% cetylpyridinium chloride (CPC) (also from Sigma Chemical Co.). The final concentration of NaCl in the wells was 0.075 M to minimize precipitation of glycopeptides. The precipitate was then collected on glass fiber filters using a cell harvester (Brandel #M-245; Brandel Co., Gaithersburg, MD) perfused with 0.075 M NaCl. The filters were then air dried and radioactivity assessed by scintillation counting.

In some experiments, the relative distribution of ^{3}H-labeled GAG in the fibroblast cell layer and supernatant was determined. In these experiments, fibroblasts were incubated under control or experimental conditions with [^{3}H]glucosamine as described. At the end of the incubation period, the supernatant was removed, the cell layer washed, and the supernatant and wash combined. The cell layer and the pooled supernatant plus wash were processed separately as described.

To determine whether alterations in [^{3}H]glucosamine incorporation represented increased GAG production per cell, fibroblasts were grown to confluence and then incubated with or without cytokine(s) as described. Cell number was then assessed with hemocytometer counting.

Chemical. To confirm the validity of isotopic incorporation as a measure of fibroblast GAG accumulation, GAGs were also assessed using chemical methods as previously described (30, 31). Cells were grown to confluence, cytokine(s) was added, the cells incubated for 72 h and pronase added as previously described. Replicate wells were then pooled, boiled in H_2O for 10 min, 200 μg of RNAase (Cooper Biomedical, Malvern, PA) (1 mg/ml in H_2O) was added and the solutions incubated at 37°C overnight. At the end of this incubation period, 100 μg of DNAase (Miles Laboratories, Elkhart, IN) (1 mg/ml in 0.05 M $MgCl_2$) was added, followed by an overnight incubation, the addition of 100 μg of pronase and an additional overnight incubation. The GAGs were then precipiated by the addition of three volumes of absolute alcohol, collected after centrifugation, resuspended, and lyophilized. The lyophilized sample was resuspended in 0.1 ml distilled water and 0.0005 ml applied to cellulose acetate strips (Beckman Instruments, Inc., Fullerton, CA) using a Beckman sample applicator. All cellulose acetate strips had been prewashed in 30% methanol. Known amounts of hyaluronic acid and sulfated GAGs (Miles Laboratories) were always run in tandem. Electrophoresis was performed in a Beckman microzone apparatus in LiCl (0.05 M), EDTA (0.01 M) adjusted to pH 8.4 at 4°C for 15 min at a constant 9 mA. The strips were then immersed in absolute ethanol-1% Tween 20 for 10 s and dried on the hydrophilic side of gel bond (BioProducts, Rockland, ME). The dried strips were immersed for 5 min while gently shaking in 10 ml of 0.01 M ammonium formate (pH 3.0) containing 2.5% polyethyleneglycol 6,000 (Fisher Scientific, King of Prussia, PA), 0.05% Tween 20 and 4×10^6 dpm ^{125}I-cytochrome *c* (sp act 0.02 μCi/μg). The strips were then serially washed in 0.01 M ammonium formate-0.05% Tween 20 (pH 3.0), 6 M urea (pH 3.4) and 0.01 M ammonium formate-Tween 20 buffer, and dried on the hydrophilic side of gel bond. ^{125}I-Cytochrome *c* binding was then assessed by autoradiography using Ortho G x-ray film (Kodak Co., Rochester, NY) in Lanex intensifying screens for 16 h at −70°C. The autoradiographs that were obtained were then evaluated in an LKB 2222-010 densitometer (LKB, Paramus, NJ).

To prepare the ^{125}I-cytochrome *c*, 300 μg of horse heart type III cytochrome *c* (Sigma Chemical Co.) was dialyzed in 0.1 M borate buffer (pH 8.0) and labeled in the presence of 60 μg Iodo Gen (Pierce Chemical Co., Chicago, IL) with 300 μCi ^{125}I-Na (Amersham Corp., Arlington Heights, IL) using the method of Fraker and Speck (32). The labeled protein was then separated from the unreacted iodine on a Sephadex G-25 PD10 column (Pharmacia Fine Chemicals, Piscataway, NJ) and cold cytochrome *c* added so that each 100 μg of protein contained 4×10^6 dpm.

Characterization of GAGs

Isotopic. To determine whether the [^{3}H]glucosamine was being incorporated into GAG, and the size and types of GAGs being produced, replicate wells were pooled, and the CPC precipitates collected by centrifugation. The precipitates were dissociated with 2 M $MgCl_2$ and the GAGs reprecipitated with three volumes of alcohol. After centrifugation, the GAGs were resolubilized in water and eluted from a Bio-gel P-10 (Bio-Rad Laboratories, Richmond, CA) column with 0.5 M NaCl with 0.02% sodium azide as described (33). Intact GAGs appear in the void volume and glycoproteins elute in the inclusion volume of this column. The void volume fractions were then pooled and reprecipitated with three volumes of alcohol. To further assess the size of the GAG moieties being produced, the CPC precipitates were resolubilized in water and eluted from a Sepharose CL-2B column (Pharmacia Fine Chemicals) in 0.006 M phosphate buffered saline (pH 7.4) supplemented with 0.2% sodium azide. The void volume of the column was determined using highly polymerized calf thymus DNA (Sigma Chemical Co.). To determine the types of GAGs being produced, the samples were sequentially incubated with specific GAG degrading enzymes. After each digestion, P-10 column chromatography was used to sepa-

rate the undigested GAGs, which appear in the void volume of the column, from the digestate that appears in the inclusion volume of the column. The void volumes were then precipitated after addition of appropriate carrier GAG and used as the substrate for the next digestion. Comparison of the pattern of ^3H elution before and after each digestion allowed determination of the percent of label incorporated into each GAG moiety. The sequence of the specific enzymes that were employed and the conditions under which they were used are as follows: (*a*) hyaluronidase (*Streptomyces hyaluronlyticus*) (Calbiochem-Behring) for hyaluronic acid determinations: 20 U in 0.02 M acetate buffer (pH 5.0) for 16 h at 56°C. This enzyme did not contain significant proteolytic activity and did not degrade sulfated GAGs in our hands and the hands of others (34). (*b*) Chondroitinase AC (Sigma Chemical Co.) for chondroitin 4 and 6 sulfate determinations: 0.25 U in 0.2 M acetate buffer (pH 6.0) for 2 h at 37°C; (*c*) Chondroitinase ABC for dermatan sulfate quantification: 0.25 U in 0.2 M acetate buffer (pH 6.0) for 2 h at 37°C; and (*d*) heparitinase (Miles Laboratories) to assess heparan sulfate: 0.1 U in 0.02 M acetate buffer (pH 6.0) for 4 h at 43°C.

Chemical. To identify the GAGs produced by unstimulated and cytokine stimulated fibroblasts, the cells were grown to confluence, incubated with or without cytokine(s), and treated with pronase, and nucleases as described. The samples and known GAG controls were then applied to individual lanes of cellulose acetate strips, electrophoresed, and stained with ^{125}I-cytochrome *c* as described. Since cellulose acetate electrophoresis successfully separates hyaluronic acid, the chondroitin sulfates, dermatan sulfate and heparan sulfate, the resulting autoradiograph allows for an accurate quantification of the GAGs in the test sample (30, 31). Confirmation that a particular band represents a given GAG moiety was also obtained by demonstrating the disappearance of the band after treatment with specific GAG degrading enzymes.

Assessment of fibroblast hyaluronate synthetase activity

The cell-free hyaluronic acid synthesizing capacity of untreated and cytokine-treated fibroblasts was assessed using modifications of techniques described by Eppig and Ward-Bailey (35) and Appel et al. (36). In brief, fibroblasts were grown to confluence and incubated with and without cytokine(s) as described. The cells were then mechanically detached, washed and resuspended in 50 mM Tris-HCl pH 7.05 containing 0.24 M sucrose. Equal numbers of cells were then removed, sonicated while on ice, and the cell debris removed by centrifugation (400 *g*) for 10 min at 4°C. Cell sonicate was then added to an equal volume of a solution containing 0.8 mM dithiothreitol, 16.6 mM ATP, 0.2 M NaH_2PO_4 (pH 6.7), 0.2 M $MgCl_2$, and 0.332 mM UDP-*N*-acetylglucosamine (Sigma Chemical Co.). UDP-[^{14}C]glucuronic acid (0.2 μCi, 320.1 mCi/mmol) (New England Nuclear, Boston, MA) was then added and the solution incubated overnight at 37°C in 5% CO_2 and air. At the end of the incubation period, carrier hyaluronic acid was added (0.05 ml, 2 mg/ml) and the samples transferred to 2.3 cm filter paper disks (Whatman Inc., Englewood Cliffs, NJ) and air dried. The filter disks were then washed with three changes of a solution containing 0.5% cetylpyridinium chloride, 0.02 M NaCl, and 0.05 M Tris (pH 7.0) for 20 min, air dried once again and the radioactivity measured by scintillation counting.

To identify the reaction products that were formed, some samples were split. Both halves were processed as described above except one was treated with streptomyces hyaluronidase before the addition of carrier hyaluronic acid. The difference in radioactivity between the samples was an index of the amount of hyaluronic acid produced.

To be sure that the differences in hyaluronate synthetase activity that were noted were due to differences in biosynthesis and not to alterations in the rate of degradation, we determined whether the cell sonicates degraded [^3H]hyaluronic acid. The ^3H-labeled GAG was prepared by incubating fibroblasts in complete medium supplemented with 10–20% FBS for 72 h followed by pronase digestion and CPC precipitation. The CPC precipitates were then collected by centrifugation, resolubilized in $MgCl_2$, precipitated with alcohol, resuspended in H_2O, and eluted from a CL-2B column. The GAGs that eluted in the void volume of the column were then precipitated with alcohol and resuspended in complete medium. Equal volumes of cell sonicate and [^3H]GAG ($\cong$ 50,000 counts total) were then incubated at 37°C for 24 h and the [^3H]GAG reeluted from the CL-2B column. GAG digestion was assessed by determining if the incubation slowed the elution of the [^3H]GAG from the column.

Results

Recombinant cytokine regulation of fibroblast GAG production. Fibroblasts incubated in complete medium incorporated modest amounts of [^3H]glucosamine into GAG. rTNF, rLT (Fig. 1), and rIFN-γ (Fig. 2) each caused a dose-dependent increase in this incorporation. Similar stimulatory effects were noted on fibroblasts incubated with or without 10% FBS. Peak stimulation was noted when fibroblasts were incubated for 72 h with 1,000 IU/ml of rIFN-γ, 20 ng/ml of rTNF or 20 ng/ml of rLT. At these concentrations, fibroblasts incorporated 140, 159, and 158%, respectively, as much [^3H]glucosamine into GAG as controls ($P < 0.01$ for all three cytokines as vs. control, paired *t* test). No stimulation of fibroblast [^3H]glucosamine incorporation was observed with rIFN-α_A, -α_D, or -β_1. Fibroblasts incubated for 72 h in 1,000 IU/ml of rIFN-α_A, -α_D, and -β_1 incorporated 94, 89, and 101% as much [^3H]glucosamine into GAG as controls ($P \leq 0.42$ for all three interferons as vs. controls, paired *t* test) (Fig. 2).

The simultaneous addition of rIFN-γ plus rTNF further increased fibroblast [^3H]glucosamine incorporation into GAG. This effect was synergistic since the stimulation caused by the cytokines in combination consistently exceeded the sum of the stimulation caused by either agent alone (Fig. 3, Table 1). Isobologram analysis (37) also indicated a synergistic interaction (data not shown). This synergistic stimulation was observed in experiments with 0 as well as 10% FBS and with and without ascorbic acid. Peak stimulation was noted when fibroblasts were incubated for 72 h with 1,000 IU/ml of rIFN-γ plus 20 ng/ml of rTNF where fibroblasts incorporated 480±47%, respectively, as much [^3H]glucosamine into GAG as controls ($P < 0.001$ rIFN-γ plus rTNF as vs. either cytokine alone, one way analysis of variance). When the concentration of one cytokine was kept constant, the stimulatory effect of the other was dose dependent (Fig. 3). In the presence of 1000 IU/ml of rIFN-γ, rTNF doses as low as 0.02 ng/ml synergistically stimulated fibroblast [^3H]glucosamine incorporation into GAG. In the presence of 20 ng/ml of rTNF, rIFN-γ doses as

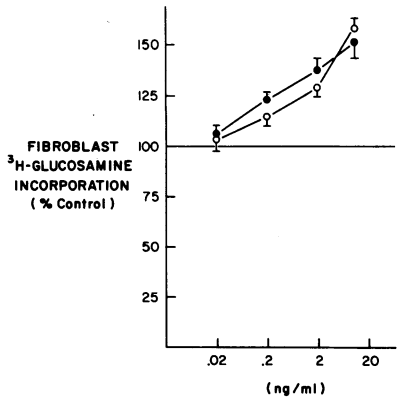

Figure 1. Demonstration of the stimulation of fibroblast GAG production by rTNF (●) and rLT (○). Fibroblasts were incubated for 72 h in serum free conditions in the presence of rTNF and rLT and [^3H]glucosamine incorporation into GAG was assessed as described. Values represent the mean±SE of 18 experiments with each cytokine.

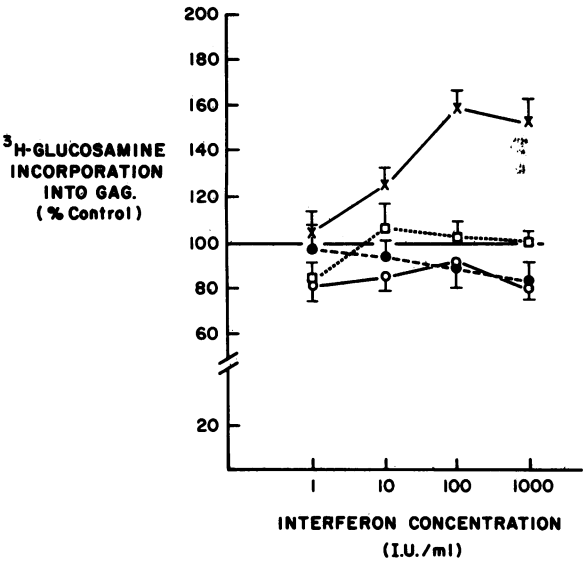

Figure 2. IFN-induced stimulation of fibroblast GAG production. Fibroblasts were incubated in the complete medium only and in complete medium supplemented with rIFN-γ (×), -α_A (●), -α_D (○), and -β_1 (□) and [^{3}H]glucosamine incorporation into GAG assessed as described. Values represent the mean±SE of 15 experiments with each cytokine.

low as 1-10 IU/ml synergistically stimulated fibroblast GAG biosynthesis. When rLT was combined with rIFN-γ, a similar synergistic stimulation was noted (Table I). In contrast, IFN-α_A, -α_D and -β_1 did not interact synergistically with rTNF or rLT. Fibroblasts cultured with IFN-α_A, -α_D and -β_1 plus rTNF or rLT incorporated as much [^{3}H]glucosamine into GAG as cells incubated with rTNF or rLT alone ($P \leq 0.4$ for all, one way analysis of variance) (data not shown).

To be sure that [^{3}H]glucosamine incorporation accurately assessed fibroblast GAG production, experiments were simultaneously performed in which GAG production was measured using chemical means (cellulose acetate electrophoresis followed by staining with ^{125}I-cytochrome *c* and autoradiography). These studies showed that rIFN-γ and rTNF increased

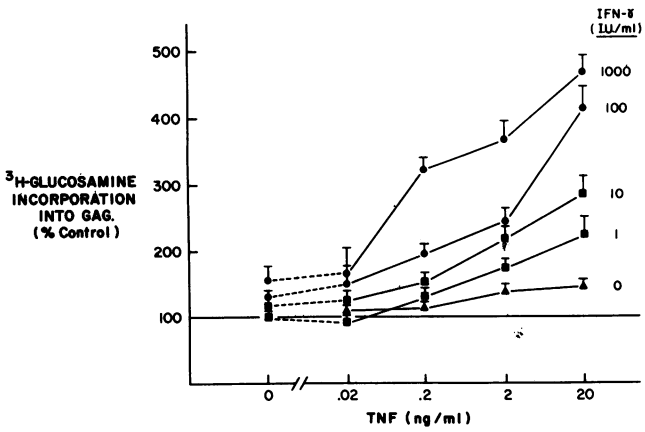

Figure 3. Synergistic stimulation of fibroblast GAG production by rIFN-γ and rTNF. Fibroblasts were incubated for 72 h in serum-free conditions in the presence of rIFN-γ and rTNF, alone and in combination. GAG production was assessed as described. Values represent the mean±SE of 12 experiments.

Table I. Cytokine Regulation of Fibroblast GAG Production

Fibroblast culture conditions*	[^{3}H]Glucosamine incorporated into GAG
	cpm/well
Medium	15,828±587
rIFN-γ	21,565±1,228
rIFN-γ + anti-IFN-γ	16,787±1,722
rTNF	29,582±1,890
rTNF + anti-TNF	15,383±358
rLT	26,987±2,143
rLT + anti-LT	15,439±1,183
rIFN-γ + rTNF	62,515±898
rIFN-γ + rTNF + anti-IFN-γ	27,978±648
rIFN-γ + rTNF + anti-TNF	21,669±1,104
rIFN-γ + rTNF + anti-IFN-γ + anti-TNF	14,377±1,331
rIFN-γ + rLT	60,499±3,103
rIFN-γ + rLT + anti-IFN-γ	27,513±1,031
rIFN-γ + rLT + anti-LT	22,404±1,444
rIFN-γ + rLT + anti-IFN-γ + anti-LT	16,416±1,611

* Fibroblasts were incubated for 72 h in complete medium only (medium) or in complete medium supplemented with rIFN-γ (1,000 IU/ml), rTNF (20 ng/ml) and rLT (20 ng/ml). The effect of these cytokines and cytokine combinations was assessed before and after incubation (37°C, 1 h) with an equivalent number of neutralizing units of the noted monoclonal antibodies.

fibroblast GAG production. They also showed that the two cytokines interacted in a synergistic fashion to augment GAG accumulation (Fig. 4). This demonstrates that the results obtained by assessing [^{3}H]glucosamine incorporation into pro-

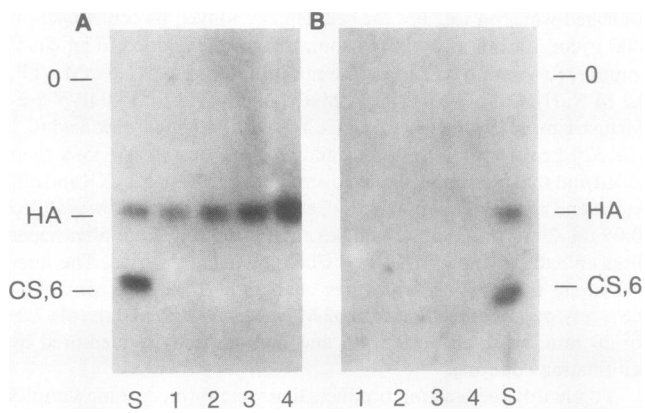

Figure 4. Chemical analysis of fibroblast GAG production. Fibroblasts were incubated with and without cytokine(s). The quantity and type of GAGs produced were then assessed using cellulose acetate electrophoresis, ^{125}I-cytochrome *c* staining and autoradiography as described. GAG analysis was performed without addition of hyaluronidase (*A*) and after incubation with hyaluronidase (*B*). Fibroblasts were incubated in complete medium only (lane *1*) and in complete medium with rIFN-γ (1,000 IU/ml) (lane *2*), rTNF (20 ng/ml) (lane *3*), and rIFN-γ (1,000 IU/ml) plus rTNF (20 ng/ml) (lane *4*). S, simultaneously run standards; HA, hyaluronic acid; CS,6, chondroitin 6 sulfate; O, origin.

nase-resistant CPC precipitable counts accurately reflect fibroblast GAG production. This also rules out the possibility that changes in intracellular glucosamine pool size were responsible for the changes in [^{3}H]glucosamine incorporation that were observed.

The stimulation of fibroblast GAG production caused by rTNF, rLT, and rIFN-γ, alone and in combination, was cytokine induced and not the result of alterations in cell number. Preincubation of each cytokine with its respective monoclonal antibody abrogated its stimulatory effect (Table I). In addition, direct cell counts did not reveal an alteration in cell number that could account for the changes observed in [^{3}H]glucosamine incorporation (Table II). The stimulation of GAG production caused by these cytokines and cytokine combinations was also not specific for supernatant or cell-associated GAG. Approximately 67% of the [^{3}H]glucosamine incorporated into GAG by the control fibroblasts was detected in their supernatants. Fibroblasts incubated in these cytokines and cytokine combinations had a similar percentage of ^{3}H labeled GAG in their supernatants (Table III), indicating that the cytokines did not alter GAG secretion.

Characterization of GAGs. To confirm that the [^{3}H]glucosamine was being incorporated into GAG, CPC precipitates were solubilized and chromatographed on a P-10 column. Greater than 95% of the [^{3}H]glucosamine in the precipitates from control cultures and cells treated with the cytokines or cytokine combinations appeared in the void volumes of these columns. In fact, a portion of the [^{3}H]GAG appeared in or near the void volume of a CL-2B column (Fig. 5). Thus, the radiolabel was incorporated into large anionic macromolecules compatible with GAG.

The types of GAGs being produced were then determined isotopically and chemically. Isotopic analysis entailed serial digestion with specific GAG degrading enzymes. These studies demonstrated that under control conditions, 82.1% of the synthesized GAG was hyaluronic acid while 4.6, 7.4, and 4.3% were chondroitin 4 or 6 sulfates, dermatan sulfate, and heparan sulfate, respectively (Table IV). They also demonstrated that incubation with rIFN-γ, rTNF, and the two cytokines in combination caused the fibroblast to produce a higher percentage of hyaluronic acid and a lower percentage of sulfated GAG moieties (Table IV). Similarly, the cytokines and cytokine combinations did not significantly alter fibroblast incorporation of $Na_2{}^{35}SO_4$ into GAG (Table V). Chemical analysis confirmed these observations. When the GAGs produced by control fibroblasts were electrophoresed and exposed to ^{125}I-cytochrome *c*, autoradiography demonstrated a prominent band corresponding to hyaluronic acid with no staining in the areas of the sulfated GAGs (Fig. 4). In addition, the cytokines and cytokine combinations increased hyaluronic acid staining without altering that for the other GAGs (Fig. 4). Thus, under all conditions, the majority of the GAG being produced by lung fibroblasts is hyaluronic acid. In addition, increased hyaluronic acid production is the major stimulatory effect of rIFN-γ and rTNF, alone and in combination.

Mechanism of cytokine stimulation of fibroblast GAG production. Experiments were performed to examine the role fibroblast cytotoxicity and fibroblast prostaglandin and protein production play in mediating the GAG regulatory effects of these cytokines. Fibroblasts incubated with rIFN-γ and rTNF (alone and in combination) excluded trypan blue dye as effec-

Table II. Comparison of Isotope Incorporation and Cell Numbers

Fibroblast culture* conditions	[^{3}H]Glucosamine incorporation‡	Cells per cm^2§
	cpm/well	
Medium	3,786±204	3.42×10^4
rIFN-γ	5,342±560	2.89×10^4
rTNF	6,599±515	3.35×10^4
rIFN-γ + rTNF	14,505±1,168	2.80×10^4

* Fibroblasts were incubated for 72 h in complete medium only (medium) or in complete medium supplemented with rIFN-γ (1,000 IU/ml), and rTNF (20 ng/ml).
‡ [^{3}H] Glucosamine incorporation into pronase resistant CPC precipitable counts as per Methods.
§ Cells per square centimeter at end of incubation period.

Table III. Distribution of [^{3}H]GAG between Cell Layer and Supernatant

Fibroblast culture conditions*	% Cell-associated‡
Medium	33±4
rIFN-γ	35±7
rTNF	34±6
rTNF + rIFN-γ	30±5

* Fibroblasts were incubated under control conditions and in the presence of rIFN-γ (1,000 IU/ml) and rTNF (20 ng/ml), alone and in combination. The supernatant was then aspirated, the cell layer washed and the [^{3}H]GAG in the supernatant and cell layer determined separately as described in *Materials and Methods.*
‡ Percent of total incorporated [^{3}H]glucosamine associated with cell layer. Values represent the mean±SE of six experiments.

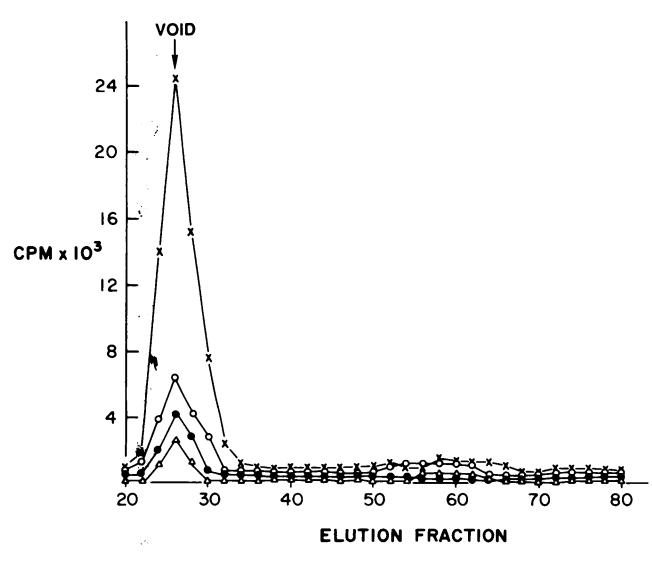

Figure 5. Sepharose CL-2B chromatography of ^{3}H-labeled GAG. Fibroblasts were incubated in complete medium only (△) and complete medium with rIFN-γ (1,000 IU/ml) (●), rTNF (20 ng/ml) (○), and the two cytokines together (×). The GAGs were isolated and eluted from a Sepharose CL-2B column as described.

Table IV. Profile of Glycosaminoglycans Produced by Adult Human Lung Fibroblasts

Culture conditions*	Percentage of ^{3}H-labeled GAG‡			
	Hyaluronic acid	Chondroitin sulfates	Dermatan sulfate	Heparan sulfate
Medium	82.1±3.1	4.9±1.3	7.7±2.1	4.6±1.1
IFN-γ	85.6±5.3	2.2±0.4	6.7±1.8	4.8±1.7
TNF	90.1±4.1	1.9±0.3	4.9±1.7	2.5±0.8
IFN-γ plus TNF	92.3±3.1§	3.3±1.1	3.3±0.4	1.7±0.5

* Fibroblasts were incubated for 72 h in complete medium only (medium) or complete medium supplemented with rIFN-γ (1,000 IU/ml) and/or rTNF (20 ng/ml) as noted.
‡ Percentage of total ^{3}H-labeled GAG susceptible to digestion with fungal hyaluronidase (hyaluronic acid), chondroitinase AC (chondroitin sulfates), chondroitinase ABC (dermatan sulfate) and heparitinase (heparan sulfate) as described in Methods. Values represent the mean±SE of eight experiments performed using fibroblast lines CCL-202 and CCL-201. In all experiments > 93% of the counts applied to the columns were recovered.
§ $P < 0.05$ as vs. media controls (paired t test).

tively as controls, suggesting that cell cytotoxicity was not an important mediator of these effects. Similarly, indomethacin (1 μg/ml) effectively blocked fibroblast prostaglandin production but did not alter fibroblast GAG production, indicating that fibroblast prostaglandin production was also not mediating these regulatory events. In contrast, protein and mRNA production appeared to be important since cyclohexamide (50 μg/ml) and actinomycin D (2.0 μg/ml) abrogated these stimulatory effects (Table VI).

To further understand the mechanism of these effects, the hyaluronate synthetase activity of sonicates from untreated and cytokine-treated cells was assessed by characterizing the ability of the sonicates to incorporate UDP-[^{14}C]glucuronic acid into CPC precipitable counts. These experiments demonstrated that rIFN-γ and rTNF each stimulated UDP-[^{14}C]glucuronic acid incorporation (Table VII). In addition, they demonstrated that the two cytokines interact in a synergistic fashion to augment fibroblast hyaluronate synthetase activity (Table VII). This assay assesses hyaluronate synthetase activity since, under all conditions, > 95% of the incorporated UDP-[^{14}C]glucuronic acid was susceptible to digestion with hyaluronidase (data not shown). In addition, the effects of the cytokines were due to augmented synthesis and not decreased degradation since sonicates from control and cytokine-treated cells did not alter the pattern of elution of [^{3}H]GAG from the CL-2B column (data not shown).

Discussion

In this study, we have analyzed the effect of rTNF, rLT, and rIFN-γ, alone and in combination, on the glycosaminoglycan production of normal lung fibroblasts. The results demonstrate that these cytokines stimulate GAG production and that rIFN-γ synergizes with rTNF and rLT to further augment GAG biosynthesis. These cytokines did not alter the distribution of GAGs between the cell layer and supernatant and their stimulatory effects were not the result of alterations in cell number. In all cases, hyaluronic acid was the predominant GAG moiety affected and its stimulation appeared to be mediated, at least partly, by the induction of cellular hyaluronate synthetase activity.

This study demonstrates that neither rIFN-α nor rIFN-β_1 stimulates fibroblast GAG biosynthesis and neither interacts with rTNF and rLT to augment GAG production. These findings are in accord with previous observations that IFN-α and -β share a receptor distinct from that of IFN-γ (38), and that IFN-α and -β interact with TNF and LT less effectively than IFN-γ in regulating a number of cellular functions (20, 22, 39).[2]

Despite their different cells of origin, rTNF and rLT had similar effects in these studies. Each stimulated GAG production and synergized with rIFN-γ to further augment GAG biosynthesis. This similarity is in keeping with prior demonstrations that these two peptides have similar functional profiles (17, 22) and may reflect their 50% amino acid homology (40) and their binding to a common receptor (41).

In these studies we used isotopic and chemical techniques to characterize the GAGs made by unstimulated and cytokine-treated fibroblasts. Both techniques demonstrated that hyaluronic acid is the major GAG produced by lung fibroblasts and that augmented hyaluronic acid accumulation is the major stimulatory effect of the cytokines and cytokine combinations. To our knowledge, this is the first demonstration of differential regulation of the production of different GAG species by mononuclear cell-derived mediators. However, differential regulation of this sort is not without precedent since Hammerman et al. described a cartilage-derived factor(s) that stimulated fetal chondrocyte hyaluronic acid production and inhibited sulfated GAG biosynthesis (3).

The mechanism by which the cytokines specifically stimulate fibroblast hyaluronic acid production is unknown. Studies from this and other laboratories have shown that TNF stimu-

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

Experiment	$Na_2{}^{35}SO_4$ (cpm/well)				[^{3}H]Glucosamine (cpm/well)			
	Medium	IFN-γ	TNF	IFN-γ + TNF*	Medium	IFN-γ	TNF	IFN-γ + TNF*
1	14,091±1,093	15,038±985	15,619±700	12,819±375	7,135±486	12,358±136	14,437±417	42,271±1,701
2	11,979±1,232	10,356±947	10,506±374	12,786±497	8,316±801	12,565±782	11,417±110	28,630±482
3	7,011±319	6,268±305	4,731±240	4,837±221	2,937±60	3,343±22	6,933±368	24,413±162

* Fibroblasts were incubated in parallel with $Na_2{}^{35}SO_4$ or [^{3}H]glucosamine for 72 h in complete medium only (medium) or with complete medium supplemented with rIFN-γ (1,000 IU/ml) or rTNF (20 ng/ml) as noted. Isotope incorporation was assessed as described in Methods.

Table VI. Effect of Actinomycin D and Cyclohexamide on Cytokine Stimulation of Fibroblast GAG Biosynthesis

	[^{3}H]Glucosamine incorporation (cpm/well)			
Culture conditions*	Medium	rIFN-γ	rTNF	rIFN-γ plus rTNF
Control	12,486±533	18,488±954	23,143±1,634	63,129±1,209
Cyclohexamide	14,413±862	14,862±611	18,052±1,395	14,187±815
Actinomycin D	8,894±433	9,009±236	7,520±252	7,050±227

* Fibroblasts were incubated for 72 h in complete medium only or in complete medium supplemented with rIFN-γ (1,000 IU/ml), rTNF (20 ng/ml) or both cytokines together. The [^{3}H]glucosamine incorporated into GAG by these control fibroblasts is compared to that of identical cultures to which cyclohexamide (50 μg/ml) or actinomycin D (2.0 μg/ml) was added.

lates fibroblast prostaglandin production (21, 23) and that prostaglandins can stimulate fibroblast GAG biosynthesis (42). However, indomethacin, while blocking fibroblast prostaglandin production, did not affect cytokine stimulation of fibroblast GAG biosynthesis suggesting that prostaglandins are not involved in this stimulatory process. On the other hand, cyclohexamide and actinomycin D abrogated the stimulation of fibroblast GAG biosynthesis caused by these cytokines. Thus, protein biosynthesis and mRNA synthesis appear to play an important role in mediating the stimulation of hyaluronic acid production caused by these cytokines. Our demonstration that cytokine induced alterations in hyaluronic acid accumulation are paralleled by changes in cellular hyaluronate synthetase activity suggests that this poorly understood enzyme (or series of enzymes) plays an important role in mediating these changes. This speculation is in accord with prior studies that suggest that this enzyme(s) can play a regulatory role (43, 44). Whether other proteins, such as the protein primer that some (but not all) investigators feel is needed to initiate hyaluronic acid production (45–47), are also involved in this regulation will require additional study. Further investigation will also be needed to determine whether the relative selectivity of the effect is due to hyaluronic acid inhibiting fibroblast production of sulfated GAGs as has been described in other systems (3, 48, 49).

Hyaluronic acid accumulation can result from increased biosynthesis or decreased degradation. Our studies demonstrate that cytokine(s) stimulation of hyaluronic acid accumulation is associated with increased hyaluronate synthetase activity in fibroblast sonicates. In contrast, the fibroblast sonicates did not contain hyaluronic acid degradative activity. This demonstrates that at least part of the stimulation of fibroblast hyaluronic acid accumulation caused by the cytokines and cytokine combinations is the result of augmented hyaluronic acid biosynthesis. However, fibroblast hyaluronidase is felt to be a lysozomal enzyme with a pH optimum of 3.0 (50, 51). Thus, these studies do not rule out the possibility that the cytokines also alter hyaluronic acid degradation.

A number of studies have shown that rapidly proliferating fibroblasts produce more hyaluronic acid and contain more hyaluronate synthetase activity per cell than quiescent cells (52, 53). This led us to speculate that the alterations in hyaluronic acid production were due to cytokine-induced alterations in cell proliferation. However, when hyaluronic acid production and cell numbers were simultaneously assessed, we did not see an alteration in cell number that could account for the changes in hyaluronic acid production. In addition, we did not see evidence of cell cytotoxicity that would allow cells to proliferate without causing an increase in cell number. These observations suggest that fibroblast hyaluronic acid production and cell proliferation can be regulated independently.

These studies demonstrate that rIFN-γ, rTNF, and rLT have important effects on the quantity and quality of GAGs produced by fibroblasts in vitro. If similarly operative in vivo, these observations have important implications for understanding the function, growth and repair of the lung and other organs. It is well documented that alterations in GAG accumulation occur during morphogenesis (10, 11) and that GAGs may play an important role in regulating cellular proliferation and differentation (3) during these processes. In the complete organ, GAGs provide cellular support and direct the movements of fluids and macromolecules (1–3). In addition, they can modulate the local inflammatory response via regulating macrophage interleukin 1 production (1) and lymphocyte proliferation (2). Thus, mononuclear cell–mediated alterations in GAG production can potentially regulate a wide array of cell and organ functions.

The findings in these studies may also have bearing on the pathophysiology of sarcoidosis, scleroderma, and rheumatoid arthritis. Unlike normal individuals, patients with sarcoidosis have increased levels of hyaluronic acid in their bronchoalveolar lavage (54) and their lung mononuclear cells spontaneously elaborate IFN-γ (55) and TNF (56). Patients with scleroderma have excessive amounts of GAG in their skin (8) and dermal fibroblasts that elaborate abnormally large amounts of hyaluronic acid in vitro (13). In addition, mononuclear cell infiltrates frequently accompany their sclerodermatous skin

Table VII. Cytokine Regulation of Fibroblast Hyaluronate Synthetase Activity

Fibroblast culture conditions*	UDP-[^{14}C]Glucuronic acid incorporation‡
	cpm
Media	174±10
rIFN-γ	239±19
rTNF	360±28
rIFN-γ±rTNF	1,051±73

* Fibroblasts were incubated for 72 h in complete media only (medium) or in complete medium plus rIFN-γ (1,000 IU/ml) or rTNF (20 ng/ml) as noted.
‡ 2.5×10^5 cells were sonicated and hyaluronate synthetase was assessed as described in Methods.

changes (57), their mononuclear cells spontaneously elaborate inflammatory mediators (58), and normal fibroblasts take on a propagatable sclerodermatous phenotype when chronically exposed to supernatants from activated mononuclear cells (9). Patients with rheumatoid arthritis have elevated serum (59) and joint space (14) levels of hyaluronic acid, and their synovial cells produce excessive amounts of hyaluronic acid when cultured in vitro. (14). In addition, activated macrophages and lymphocytes comprise a significant number of the cells in the synovia of these patients (60), and IFN-γ has been shown to abnormally accumulate in their synovial fluid (60, 61). In these disorders, abnormal GAG accumulation is seen in association with activated mononuclear cells and abnormal cytokine elaboration. The GAG abnormalities that are seen in these diseases may result from abnormal mononuclear cell regulation of fibroblast function. Our data suggest that IFN-γ, TNF, and/or LT may be responsible for mediating these mononuclear cell effects.

TNF was initially identified in the sera of mice treated with BCG and endotoxin (62). LT was independently identified in culture supernatants from activated T lymphocytes (63). Initial interest in these moieties centered around their ability to kill malignant cells. It is now clear that these moieties are more than tumor cytotoxins and have wide ranging regulatory effects (20–26).[2] Our studies demonstrate that TNF and LT, particularly when associated with IFN-γ, are also important regulators of fibroblast GAG, particularly hyaluronic acid, production and thus, are potentially important modulators of the human fibrotic response.

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