Stimulation of Glycosaminoglycan Synthesis in Cultured Human Dermal Fibroblasts by Interleukin 1

Induction of Hyaluronic Acid Synthesis by Natural and Recombinant Interleukin 1s and Synthetic Interleukin 1β Peptide 163-171

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Abstract

Hyaluronic acid (HA) is believed to play a critical role in wound healing and in morphogenesis. Factors controlling the production of HA by fibroblasts in normal and pathological states are not completely understood. In this report we have observed that natural human interleukin (IL-1)1β and human recombinant (hrIL)-1α and β are potent stimulators of HA production by fibroblasts in vitro. Hyaluronic acid is the major species of glycosaminoglycan (GAG) stimulated by IL-1 in fibroblasts. PGE_2 does not appear to be involved directly in this IL-1 effect on fibroblasts, but stimulation of HA production by IL-1 is dependent on protein synthesis. The synthetic human IL-1β peptide 163-171 (Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys), which has been previously shown to stimulate thymocyte proliferation but not fibroblast PGE_2 production, is also able to stimulate fibroblast HA production. The synthesis and secretion of IL-1 by mononuclear phagocytes at sites of inflammation and immune reactions in vivo could potentially serve as a signal for fibroblasts to synthesize HA, which in turn could serve to facilitate and modulate reparative and immune processes by virtue of its ability to alter cell-cell, cell matrix, and cell-membrane receptor interactions.

Introduction

Rapid tissue generation is exhibited in embryonic development and wound healing. Increased production of hyaluronic acid (HA)[1] is an early event in connective tissue reparative processes associated with wound healing in mammals and limb regeneration in amphibians (1–4). During embryonic development, cellular migration has been correlated with elevations of HA concentrations in chick cornea and mouse neural crest (4–6).

HA is synthesized early during the course of an inflammatory reaction and is later degraded by fibroblasts during the healing phase of inflammation at a time when synthesis of sulfated glycosaminoglycans (GAGs) is increasing (7). Mechanisms involved in controlling this resynthesis of GAGs are not completely understood. In the present report, we have observed that natural human IL-1β, human recombinant (hr) IL-1α and β and synthetic human IL-1β peptide 163-171 stimulate the synthesis of HA by dermal fibroblasts in vitro. The potential importance of the observation in postinflammatory tissue repair is discussed.

Methods

Fibroblast cultures. Cultures of human fibroblasts were established from infant foreskin explants as previously described in maintenance medium consisting of Eagle's minimum essential medium (MEM) supplemented with nonessential amino acids, ascorbic acid (50 μg/ml), $NaHCO_3$ and Hepes buffers (pH 7.2), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1 μg/ml), and 9% heat-inactivated FCS (8).

Leukocyte cultures. Mononuclear leukocytes (MNL), separated by the Ficoll-Hypaque technique from venous blood of normal donors were cultured at a density of 3×10^6 cells/ml of serum-free RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and Hepes buffer (PSH) (Gibco Laboratories, Grand Island, NY) for 72 h (9). Cultures were stimulated with PHA, 1 μg/ml (Burroughs Wellcome, Research Triangle Park, NC). Culture supernatants were stored at −70°C for up to 3 wk until they were tested for their ability to stimulate fibroblast GAG production.

Enzymatic analysis of GAGs. Enzymatic analysis of the species of GAGs produced by fibroblasts was performed as previously described (1). The GAGs were labeled by incubating confluent fibroblasts (35 mm petri dishes) with 25 μCi/ml [^{3}H]acetate (sp act 3.4 Ci/mmol; New England Nuclear, Boston, MA) and 10% (by volume) test sample for 24 h. After pronase digestion, each sample was divided into eight aliquots (0.5 ml each), suspended in enriched Tris buffer (0.05 M Tris, 0.03 M sodium acetate, 0.04 M NaCl, and 100 μg/liter BSA, at pH 7.2), and incubated at 37°C overnight with either buffer alone, *Streptomyces* hyaluronidase (100 TRU/ml; Calbiochem-Behring Corp., La Jolla, CA), an enzyme that degrades only HA; chondroitinase AC II (3.1 U/ml), an enzyme that degrades both HA and CS; or with chondroitinase ABC (2 U/ml), an enzyme that degrades HA, CS, and DS (1, 10, 11). The chondroitinase enzymes were obtained from Miles Laboratories, Elkhart, IN. After incubation, the samples were boiled to inactivate the enzymes, and carrier GAG (250 μg HA, and 250 μg CS) was added. The undigested GAGs were then precipitated with 5 mg

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1. *Abbreviations used in this paper:* CS, chondroitin sulfate; CTAB, cetyl trimethyl ammonium bromide; DS, dermatan sulfate; Δ-Di-OS, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; Δ-Di-4S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepy-ranosyluronic acid)-4-*O*-sulpho-D-galactose; Δ-Di-6S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose. GAGs, glycosaminoglycans; GGBS, 0.015 M glycylglycine/0.14 M NaCl at pH 7.2; HA, hyaluronic acid; PSH, penicillin (100 U/ml), streptomycin 100 μg/ml, and Hepes buffer; TIMP, tissue inhibitor of metalloproteinase.

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cetyl trimethylammonium bromide (CTAB), washed with enriched Tris buffer, solubilized, and counted. Subtraction of the labeled GAGs resistant to *Streptomyces* hyaluronidase from total CTAB-precipitable radioactivity determined the radioactivity in HA. Subtraction of labeled GAG resistant to chondroitinase AC II from the *Streptomyces* hyaluronidase resistant counts determined the radioactivity in CS. Similarly, the fraction of radioactivity found in the DS fraction was determined by subtracting chondroitinase ABC resistant labeled GAG from chondroitinase AC-resistant labeled GAG (1).

HA production assay. Initial studies indicated HA levels were higher in the medium than in fibroblast cell layers after IL-1 stimulation. In light of these data and to facilitate analysis of large numbers of samples, cells were routinely cultured in Falcon 3008 24 well plates, and only the fibroblast culture supernatants were assayed for HA. After 72 h culture, medium was removed from each well, replaced with 400 μl of maintenance medium containing 2.5% FCS, and cultures were incubated for an additional 24 h. Medium was then removed from each well and was replaced with 400 μl maintenance medium supplemented with 2.5% FCS and 25 μCi/ml [^{3}H]acetate. Control or test samples (100-μl aliquots) were then added to appropriate wells, and fibroblasts were cultured for an additional 24 h. Column fractions were tested in duplicate. All other samples were tested in triplicate.

Fibroblast culture supernatants from duplicate or triplicate wells were harvested, pooled, boiled to inactivate endogenous endoglycosidases, and incubated for 16 h (56°C) with protease (1 mg/ml). The reaction was stopped by boiling for 3 min. Samples were dialyzed extensively at 4°C against 0.2 M acetic acid and finally against GGBS to remove free [^{3}H]acetate. Aliquots (100 μl) of each dialyzed supernatant were added to microfuge tubes (Beckman Instruments, Inc., Palo Alto, CA). To one-half of the tubes for each supernatant, 20 μl enriched Tris/HCl buffer, pH 7.2 alone or containing *Streptomyces* hyaluronidase (0.5 TRU) was added. Samples were incubated in a humidified atmosphere at 37°C for 16 h. After incubation, the samples were boiled to inactivate the enzyme, and 100 μl (21.5 μg) hyaluronate was added as carrier to each tube with mixing. Undigested GAGs were precipitated by addition of 2% CTAB in water (250 μl) for 20 min at room temperature, pelleted by microfuge centrifugation (Beckman), and 200 μl of supernatant was removed and added to scintillation vials containing 5 ml Hydrofluor (National Diagnostics, Somerville, NJ). Radioactivity in each sample was measured by scintillation counting. Labeled HA content of each replicate, expressed as counts per minute, was calculated by the following formula: HA content = cpm of aliquot treated with hyaluronidase − cpm aliquot of untreated sample. All statistical analyses were performed by use of the two-sample Student's *t* test.

Analysis of unsaturated disaccharides. GAGs were isolated from fibroblast culture supernatants as previously described (12), identified, and quantitated by isolation of the unsaturated disaccharide degradation products resulting from chondroitinase ABC digestion. 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (Δ-Di-OS) is derived primarily from HA and to a lesser extent from unsulfated chondroitin, while 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose (Δ-Di-4S) and 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyronosyluronic acid)-6-*O*-sulpho-D-galactose (Δ-Di-6S) are products from degradation of chondroitin and dermatan sulfates (13). The disaccharides were separated on a carbohydrate analysis column (P/N 84038; Waters Associates, Inc., Milford, MA) and monitored at 230 nm as previously described (13). The disaccharides were identified and quantitated by comparing the elution profiles of the supernatants with that of known Δ-Di-OS, Δ-Di-4S, and Δ-Di-6S standards (Seikagaku Fine Biochemicals, Tokyo, Japan).

Quantitation of HA degradation. Aliquots (200 μl) of the fibroblast culture supernatant containing labeled GAGs ([^{3}H]acetate) were dispensed into wells of multiwell plates containing confluent fibroblast monolayers and 250 μl of maintenance medium containing 5% FCS. PHA-MNL supernatant, or RPMI 1640 containing 1 μg/ml PHA in 50-μl aliquots were separately added to six different wells. After 24 h incubation, the supernatants were harvested. The cell layers, after being washed three times with PBS, were trypsinized, harvested, and pelleted by centrifugation. The cells from each well were resuspended in 400 μl maintenance medium. The cell suspensions and harvested supernatants were incubated with protease (1 mg/ml) at 56°C for 5 h and dialyzed against water to remove digested radioactivity. HA levels were then quantitated by the hyaluronic acid production assay described above.

Conventional molecular sieve chromatography. Columns 1.5 cm in diameter and 100 cm in length containing Sephadex G-200 or G-100 were used to fractionate supernatants from cultures of MNL stimulated by PHA as previously described (8).

Thymocyte proliferation assays. IL-1 activity was quantitated by measuring the uptake of tritiated thymidine ([^{3}H]TdR) by thymocytes in direct mitogenic or comitogenic assays as previously described (14, 15).

Recombinant IL-1α and β. Purified human recombinant (hr) IL-1α and β were purchased from Genzyme Corp. (Boston, MA). These preparations each contained 45.5 thymocyte-stimulating units/ng of IL-1. HrIL-1α and β correspond to the pI 5 and pI 7 forms of IL-1, respectively, and have been previously described in detail by March and co-workers (16).

Isolation of natural IL-1β. Natural IL-1β (pI 7.0) was isolated from supernatants of cultures of monoblasts from a patient with monocytic leukemia as previously described (17). Briefly, harvested supernatants containing IL-1 were subjected to diafiltration, ultrafiltration and isoelectric focusing (IEF). Fractions generated by isoelectric focusing in the 6.8–7.2 pH range containing IL-1 activity were pooled, dialyzed at 4°C against large volumes of 0.9% NaCl, and sterilized by filtration through 0.2-μm polycarbonate membrane filters with 0.2-μm pores (Bio-Rad Laboratories, Richmond, CA).

Anti-hrIL-1α and β antibodies. Purified rabbit anti-hrIL-1α and anti-hrIL-1β immunoglobulin were obtained from Genzyme. These antibody reagents were raised against purified hrIL-1α and β and neutralize the biological activity of hrIL-1α and β, respectively. They have highly specific immunological reactivity with human IL-1α and β, respectively, but do not bind to IL-2, IL-3, IL-4, colony stimulating factor, interferon γ or tumor necrosis factor.

High performance liquid chromatography. Natural IL-1β isolated by IEF was subjected to analytical HPLC. Molecular sieve chromatography was performed using two TSK 125 protein analysis columns (Bio-Rad) connected in tandem. The columns were equilibrated and were run with GGBS.

DNA quantitation. DNA content of fibroblast monolayers was measured by the method of Kissane and Robins (18).

Measurement of PGE_2. PGE_2 was extracted from fibroblast culture supernatants and measured by a previously described radioimmunoassay technique (19).

Synthesis of human IL-1β Peptide 163-171. The nonapeptide, Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys, representing residues 163-171 of the deduced human IL-1β sequence (16) and an unrelated decapeptide Leu-Ala-Glu-Leu-Tyr-Val-Arg-Glu-His-Leu were synthesized by the solid-phase method of Merrifield with the aid of a Beckman (model 990) automated peptide synthesizer (20). The peptides were purified by gel filtration and reverse phase HPLC (21). The amino acid composition of the peptides was determined by use of a Beckman 121 MB automatic amino acid analyzer (22). The amino acid sequence was confirmed by automatic Edman degradation using a Beckman 890 M System (23).

Results

Stimulation of fibroblast HA production by natural IL-1. Partially purified (dialfiltration, ultrafiltration, and IEF) natural IL-1β from monocytic leukemia cells stimulated HA production and thymocyte proliferation by fibroblasts in a dose-dependent manner (Fig. 1). This partially purified natural IL-1β

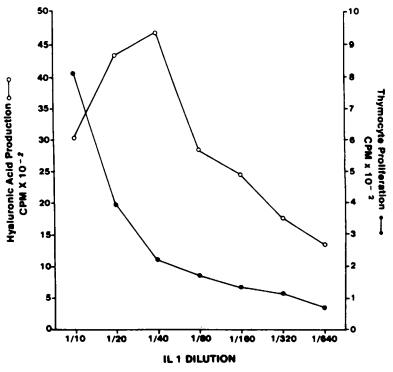

Figure 1. Natural human IL-1β prepared by diafiltration, ultrafiltration, and isoelectric focusing of supernatants from cultures of monoblasts from a patient with monocytic leukemia was tested at the dilutions indicated for its ability to stimulate thymocytic proliferation and fibroblast HA production as described in Methods. Standard errors for thymocyte proliferation and HA production assays were < 15% and 20%, respectively, of each mean value. PBS control for fibroblast HA production and thymocyte proliferation were 802±64 and 81±10 cpm, respectively.

preparation was fractionated by analytical gel filtration-HPLC, and column fractions were tested for their ability to stimulate fibroblast HA production and thymocyte proliferation (Fig. 2). The two biologic activities coeluted from the tandem TSK 125 columns, further suggesting that the factor stimulating HA production by fibroblasts was IL-1 (Fig. 2).

Additional studies were performed to further characterize the effect of IL-1 on HA and other GAG synthesis by fibroblasts. Since such experiments required large amounts of IL-1, we used supernatants from cultures of PHA-stimulated MNL and partially purified MNL or monocytic leukemia cell-derived IL-1 in these studies. Two lines of evidence indicated that most of the HA stimulating activity in supernatants from PHA stimulated MNL cultures could be attributed to IL-1. In preliminary studies, we found that the HA- and murine thymocyte-stimulating activities consistently eluted in the same fractions from conventional gel filtration columns, or gel filtration- and ion exchange-HPLC columns (data not shown). In addition, ~ 81 to 54% of the HA stimulating activity in supernatants from cultures of PHA-stimulated MNL was blocked by polyclonal antibodies to hrIL-1α and β (Table I). While these data suggest that IL-1 is the major HA-stimulatory factor produced by PHA-stimulated MNL, they do not exclude the possibility that other factors or cytokines produced by PHA-stimulated MNL may potentiate IL-1 effects on HA production by fibroblasts.

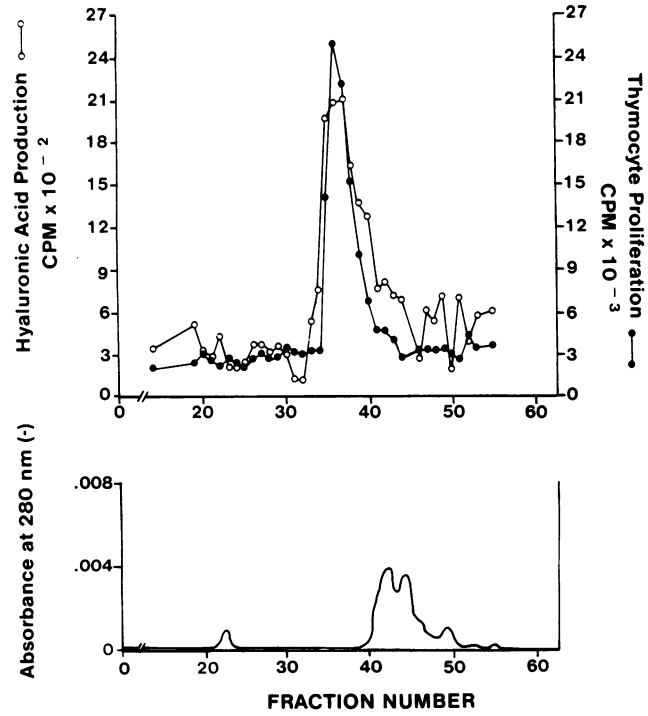

Figure 2. Natural human IL-1β prepared as described in Fig. 1 was fractionated by gel filtration-HPLC on a TSK 125 protein analysis column and fractions were tested for their ability to stimulate thymocyte proliferation and fibroblast HA production.

*Table I. Inhibition of HA Stimulating Activity in PHA-MNL Supernatant by Anti-IL-1 Antiserum**

Condition	[^{3}H]Hyaluronic acid
PHA-MNL Supernatant 1	9,249±825
PHA-MNL Supernatant 1 + Anti-hrIL-1α and Anti-hrIL-1β	1,746±59
PHA-MNL Supernatant 2	13,339±457
PHA-MNL Supernatant 2 + Anti-hrIL-1α and Anti-hrIL-1β	6,130±189
RPMI 1640 + Anti-hrIL-1α and Anti-hrIL-1β	740±29
RPMI 1640	693±54

* Supernatants (150 μl) from 72 h culture of PHA-stimulated MNL from two normal volunteers were incubated at 4°C for 16 h with 100 ml (100 μg), each, of rabbit anti-human hrIL-1α and β immunoglobulin (Genzyme). As a control, RPMI 1640 (150 μl) was incubated with 100 ml (100 μg) of each of the antibodies. Antibody-treated and untreated PHA-MNL supernatants, RPMI 1640 and antibody control were then added (100-μl aliquots) in triplicate to wells of Falcon 3008 multiwell plates containing confluent fibroblasts and [^{3}H]acetate for 24 h. Supernatants were then assayed for [^{3}H]HA content as described in Methods. Results are expressed as mean counts per minute±SEM.

Since the incorporation of [^{3}H]acetate, used in the assay to measure HA production by fibroblasts could theoretically be influenced by the rate of acetate transport and by fluctuations in precursor pool size, GAGs were also measured by the amounts of unsaturated disaccharides released after treatment of supernatants from fibroblast cultures with chondroitinase ABC (Table II). In three consecutive experiments, we found that supernatant from cultures of peripheral blood MNL stimulated previously by PHA consistently caused a significant increase in Δ-Di-OS levels when added to confluent fibroblast cultures for 72 h (Table II). Levels of Δ-Di-4S and Δ-Di-6S were also significantly increased, although to a lesser degree, after stimulation of fibroblasts with the PHA-MNL supernatant in two of three and one of three experiments, respectively (Table II). This suggests that IL-1 stimulated production predominantly of HA rather than of other GAGs. The increase in GAG production could not be attributed to a stimulation of fibroblast growth since fibroblast numbers in the cell monolayers of control and experimental dishes were not statistically different after the 72-h incubation period (Table II). In addition, the increased production of HA by fibroblasts exposed to PHA-MNL supernatant could not be attributed to diminished degradation of HA since the levels of [^{3}H]HA were found to be similar in cell layers and medium from cultures of fibroblasts

Table II. Production of Unsaturated Disaccharides by Fibroblast Cultures

Additions to fibroblast cultures*	Δ-Di-OS	Δ-Di-4S	Δ-Di-6S	Fibroblast Nos.
		mean μg/dish±SEM		*per 100 mm dish × 10^{-6}*
		Experiment 1		
PHA-MNL supernatant	12.9±1.2	0.6±0.1	2.2±0.1	1.26±0.06
RPMI 1640	2.9±0.3	0.3±0.1	1.5±0.1	1.35±0.23
P value	<0.0005	<0.01	<0.005	NS
		Experiment 2		
PHA-MNL supernatant	48.43±6.6	9.65±0.9	5.78±0.4	1.29±0.05
RPMI 1640	33.54±1.5	6.98±0.3	4.99±0.4	1.33±0.06
P value	<0.05	<0.025	NS	NS
		Experiment 3		
				per 12 wells × 10^{-5}
PHA-MNL supernatant	15.0±1.1	0.94±0.2	1.62±0.1	5.74±1.03
RPMI 1640	3.9±0.3	0.80±0.3	1.5±1.0	7.15±1.22
P value	<0.025	NS	NS	NS

* PHA-MNL supernatant (10% vol:vol) was added to separate triplicate 100 mm diameter petri dishes (experiment 1 and 2) or to three groups of 12 16 mm diameter wells of multiwell plates (experiment 3) containing confluent fibroblasts before or (as a control) after 72 h incubation at 37°C in a humidified atmosphere containing 5% CO_2, after which the cultured supernatants from each dish or group of 12 wells were individually processed, and levels of unsaturated disaccharides were quantitated as described in Methods. Fibroblasts present in each dish or per 12 wells after the 72 h incubation were harvested by trypsinization and counted in a hemacytometer. Levels of unsaturated disaccharides present in fibroblast cultured supernatants after 72 h incubation with PHA-MNL supernatant were compared with the levels of control fibroblast supernatant and analyzed by the two-sample Student's *t* test. Different and fibroblast lines batches of PHA-MNL supernatant were used in each experiment.

exposed to PHA-MNL supernatant and PHA-RPMI (data not shown).

Additional studies were performed with IL-1 to assess its effect on production of other cellular and secreted GAGs. Fibroblasts were cultured for 24 h with either column buffer (GGBS) or IL-1 containing column fractions, divided into medium and cell layer portions, and the distribution of [^{3}H]-acetate incorporation into HA, CS, DS, and other radioactive polyanions was determined. In the medium, the level of incorporation into HA induced by a column fraction containing IL-1 was four to five times the level seen with column buffer ($P < 0.0005$). This effect was also seen in the cell layer ($P < 0.05$), but was much less pronounced (Table III). No other class of GAG was stimulated to a significant extent (Table III). Analysis of GAGs in medium from fibroblasts exposed to crude PHA-MNL supernate yielded a similar result (Table III). These data are consistent with the results obtained by chemical analysis of GAG disaccharides (Table II).

Stimulation of fibroblast HA production by recombinant hrIL-1α and β. Results presented thus far strongly suggest that IL-1 could stimulate HA synthesis by fibroblasts in culture. To obtain more definitive proof that this was true, and to further

*Table III. Incorporation of [^{3}H]Acetate into Fibroblast GAGs**

Compartment	Treatment	Hyaluronic acid	Chondroitin sulfate	Dermatan sulfate	Other GAGs
		Experiment 1			
Medium	IL-1 fraction	1,803±121 (<0.0005)	115±1 (NS)	176±16 (NS)	75±20 (NS)
	Column buffer	378±8	122±8	122±16	44±10
Cells	IL-1 fraction	512±40 (<0.05)	165±100 (NS)	67±58 (NS)	285±19 (NS)
	Column buffer	364±58	150±14	9±20	253±18
		Experiment 2			
Medium	PHA-MNL supernatant	6,931±51 (<0.001)	389±153 (NS)	278±35 (NS)	ND
	Control supernatant	1,619±65	638±81	405±159	ND

* In Exp. 1, fractions from Sephadex G-200 fractionation of supernatant from PHA stimulated MNL containing IL-1 and HA stimulating activity (eluting between 10–25 K molecular weight fractions) were pooled (40 ml), concentrated by ultrafiltration (Amicon, UM 2 membrane) to a volume of 3 ml, and subjected to additional gel filtration on a column (2 cm diameter × 100 cm length) of Sephadex G-50. Aliquots of an undiluted active fraction and a control fraction (column buffer, GGBS) were added in triplicate at 10% final volume to culture dishes (35 mm diameter) containing confluent fibroblasts, and medium and cell layer GAGs were determined as described in Methods. Results are expressed as mean counts per minute±SEM.

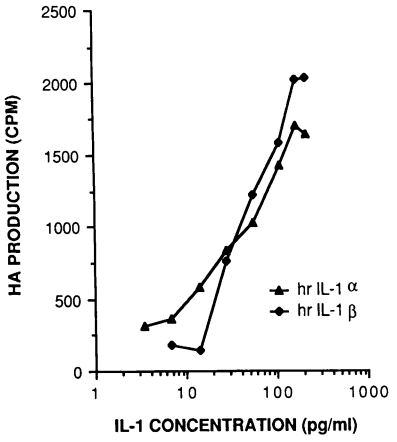

Figure 3. HrIL-1α and β were diluted in PBS and tested at the concentrations indicated for their ability to stimulate fibroblast production of HA as described in Methods. Standard errors were < 20% of each mean value. PBS control for fibroblast HA production was 285±32 cpm.

characterize this response to IL-1, we performed additional experiments with hrIL-1α and β. Both hrIL-1s stimulated HA production in a dose-dependent manner (Fig. 3) and at concentrations that triggered murine thymocyte proliferation (Fig. 4). We observed that hrIL-1α and β were usually of similar potency with respect to stimulation of HA production as illustrated in Fig. 3. However, when we examined 12 consecutive fibroblast lines, we found that two lines produced more HA in response to hrIL-1α and one line produced more HA in response to hrIL-1β, with the remaining lines responding similarly to either IL-1 preparation (data not shown).

Natural and recombinant IL-1 preparations have been shown to stimulate growth of subconfluent fibroblasts in vitro (24–28). Although confluent fibroblasts were used in studies to measure HA production, we wanted to exclude the possibility that the recombinant IL-1 preparations were merely increasing HA production by expanding the numbers of fibroblasts per well. Neither IL-1 preparations stimulated growth of confluent fibroblasts under the conditions of the assay (data not shown). These data provide definitive proof that both IL-1α and β stimulate fibroblast HA production.

Effect of inhibition of PGE_2 and protein synthesis on IL-1-induced HA production. Studies with natural and recombinant IL-1 have shown that it stimulates production of PGE_2 by human fibroblasts and adherent rheumatoid synovial cells (24, 25, 28–30). To determine whether stimulation of PGE_2 synthesis by IL-1 was linked to its stimulation of HA production, we measured HA production in IL-1-treated cultures in the presence and absence of indomethacin. Both hrIL-1α and β stimulated PGE_2 and HA production (Table IV). Addition of indomethacin to fibroblast cultures completely blocked the increased PGE_2 production induced by the IL-1s. Indomethacin also lowered basal as well as post-IL-1 treatment levels of HA, but it did not alter the magnitude of stimulation of HA production by hrIL-1α and β (Table IV). In five additional experiments in which PGE_2 was completely inhibited by indomethacin, piroxicam, or ibuprofen, we also observed no inhibition of IL-1 stimulation of HA production by fibroblasts (data not shown). These results suggest that products of the cyclooxygenase pathway of arachidonic acid metabolism are not involved in the mechanisms by which IL-1 stimulates HA production.

In an effort to determine whether protein synthesis was essential for IL-1 to exert its effect, in two separate experiments we cultured fibroblasts with cycloheximide (7.5 μg/ml) and measured HA production in response to IL-1 treatment. In both studies, cycloheximide completely blocked the effect of

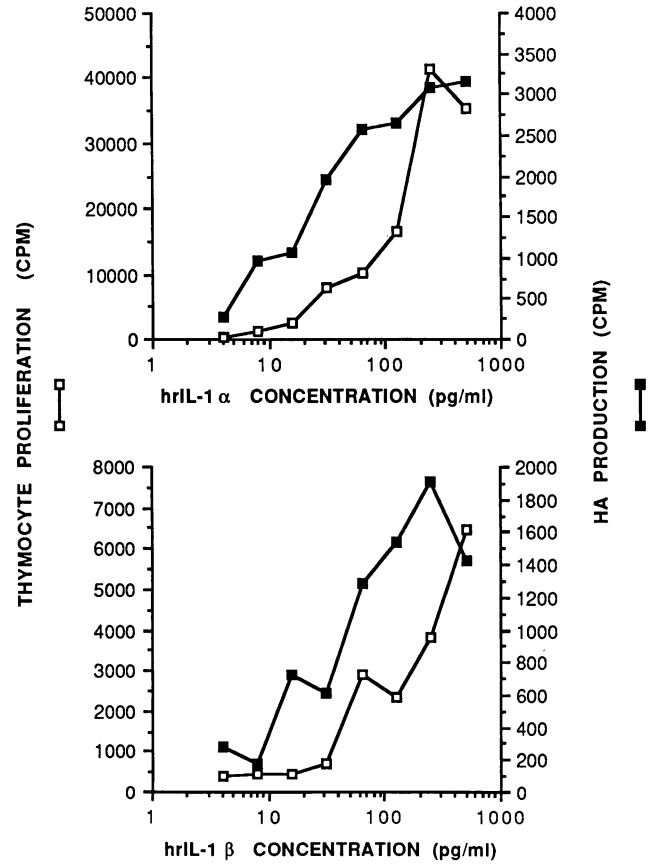

Figure 4. Human recombinant IL-1α and β were added at various concentrations to cultures of the same fibroblasts for measurement of HA production and to cultures of murine thymocytes to determine their mitogenic effects as described in Methods. Standard errors were < 20% of the mean value. PBS control for fibroblast HA production and thymocyte proliferation were 286±18 and 390±24 cpm, respectively.

*Table IV. Effect of Indomethacin on hrIL-1α and β-Induced PGE_2 and HA Production**

Condition	PGE_2 production		[^{3}H]Hyaluronic acid	
	With indomethacin	Without indomethacin	With indomethacin	Without indomethacin
	mean pg/well±SEM		*mean cpm/±SEM*	
Fibroblasts + PBS	28±3.6	33±0.2	957±55	1,493±192
Fibroblasts + hrIL-1α	30±4.7	5,547±1,419	2,160±33	3,009±310
Fibroblasts + hrIL-1β	26±2.5	193±21.1	2,177±101	2,669±120

* Indomethacin (1 mg, Sigma Chemical Co.) was solubilized in DMSO (0.1 ml DMSO with 100 mM $NaHCO_3$). The control consisted of DMSO and 100 mM $NaHCO_3$. All further dilutions were made in PBS. Fibroblasts were preincubated with indomethacin (1 μg/ml) or control for 6 h at 37°C. hrIL-1 α or β (each at a concentration of 40 pg/ml) were added to the cultures without further changes of medium, and cultures were incubated for 19 h at 37°C. Each value represents the mean of triplicate wells.

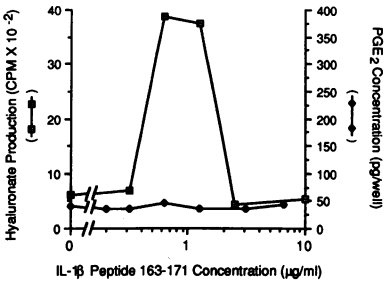

Figure 5. Synthetic human IL-1β peptide 163-171 was tested at different concentrations for its ability to stimulate production of HA and PGE_2 by a single fibroblast line. Confluent fibroblasts in multiwell plates were placed in maintenance medium (2.5% FCS) with or without the peptide for 24 h and HA and PGE_2 were quantitated in triplicate wells as described in Methods. Standard errors were < 20% of each mean value.

IL-1 on HA production, suggesting that active protein synthesis was required (data not shown).

Effect of IL-1β peptide 163-171 on fibroblast HA and PGE_2 production. Recently, it has been reported that a short synthetic fragment of human IL-1β containing residues 163-171 (Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys) stimulated murine thymocyte proliferation but did not stimulate fibroblast PGE_2 production or induce fever in mice (31). We synthesized this peptide and tested its effect in the same experiment on fibroblast HA and PGE_2 production. The peptide could induce HA but not PGE_2 production (Fig. 5). The dose-response curve for HA production was bell-shaped with optimal stimulation of HA production occurring with peptide concentrations between 0.6 and 1.2 μg/ml (Fig. 5). Similar bell-shaped dose-response curves were obtained on the two occasions this experiment was repeated with two different fibroblast lines (data not shown). Fibroblast numbers were not significantly different in control and peptide-treated cultures (data not shown). In addition, we have not been able to induce proliferation of subconfluent fibroblasts with this peptide (data not shown). As an additional control, we found an unrelated but similarly charged decapeptide (Leu-Ala-Glu-Leu-Tyr-Val-Arg-Glu-His-Leu) was not able to stimulate HA production by fibroblasts (data not shown).

Antoni et al. found that higher concentrations (175 μg/ml or greater) of this peptide were required to stimulate murine thymocyte proliferation (31). Assuming a maximal response by IL-1β peptide 143-171 in stimulating HA production at 1 μg/ml and of hrIL-1β at 0.2 ng/ml, the peptide is ~ 2,000-fold less potent on a weight basis and 100,000 less potent on a molar basis than recombinant IL-1β.

Discussion

Natural human IL-1β, hrIL-1α and β were found to be potent stimulators of HA production. Synthetic human IL-1β peptide 163-171 also stimulates fibroblast HA production but had no effect on PGE_2 synthesis. HA is the major species of GAG synthesized by dermal fibroblasts in vitro, and IL-1 was found to increase its production to a greater extent than other GAGs. Active protein synthesis is required for IL-1 to exert this effect, but PGE_2 does not appear to play a significant role in IL-1 stimulation of HA production by fibroblasts.

Whiteside et al. have previously reported that 50,000- and 15,000-D factors from gel filtration of supernatants from human MNL stimulated with concanavalin A increased GAG production by fibroblasts and stimulated murine thymocyte proliferation (32). These investigators obtained conflicting results with human and rabbit IL-1 with regards to stimulation of GAG production by fibroblasts in that a highly purified commercial preparation of human IL-1 did not stimulate GAG production under conditions employed in their study, while a partially purified preparation of rabbit IL-1 did stimulate GAG production (32).

Hamerman and Wood previously reported that a partially purified preparation of human IL-1 from monocytes and synovial cell cultures stimulated synovial cells to synthesize increased quantities of HA but not of sulfated GAGs (33). Synovial cells used in their study were from arthritic patients undergoing total joint replacement (33). Such synovial cell cultures are composed of a heterogeneous population of fibroblast- and macrophage-like cells that are morphologically and metabolically very different from dermal fibroblasts (34–36). In studies using synovial cells, it is often difficult to directly attribute a given effect to a particular mediator since cell-cell interactions involving release of other mediators are likely to occur. Yaron et al. recently reported that hrIL-1β also stimulated adherent rheumatoid synovial cells to produce increased quantities of GAGs (37). The species of GAGs were not well characterized, and hrIL-1α effects on synovial cell GAGs were not assessed in that study (37).

Fibroblasts are a major target cell for IL-1. Human gingival and murine dermal fibroblasts have been found to have more receptors for IL-1 than a variety of different lymphoid cells, including murine thymocytes, spleen and lymph node cells, and human MNL, T cell, and lymphoma lines (38, 39). IL-1 receptors on human embryonic lung fibroblasts have been reported to have high binding affinity for natural human IL-1α and β (40). Results obtained with these embryonic lung fibroblasts support the existence of a single population of IL-1 receptors on these cells (40). This would suggest that conserved homologous amino acid sequences of the two species of IL-1 may be involved in binding to the receptor. The fact that the dose response curves for hrIL-1α and β stimulation of fibroblast HA production are virtually identical is compatible with the notion that conserved regions of hrIL-1α and β bind to the same population of receptors on fibroblasts and that the active site on both molecules responsible for triggering HA production is also conserved.

Interaction of IL-1 with receptors on fibroblasts would be expected to affect several fibroblast functions. In addition to stimulating HA and PGE_2 production, IL-1 has also been found to stimulate growth of subconfluent fibroblasts and also production of collagenase, collagen, and TIMP in vitro (25–31, 41, 42). Just which amino acid sequences of IL-1α and β are involved in the binding of IL-1 to its receptors are unknown at present. Our data show that at least one fibroblast function (HA production) can be triggered by the sequence contained in IL-1β between residues 163-171. It is intriguing that neither PGE_2 production nor growth of subconfluent fibroblasts are stimulated by this same sequence. This would suggest that perhaps another sequence(s) is(are) involved in stimulation of proliferation and synthesis of PGE_2. The effect of synthetic IL-1β peptide 163-171 on other fibroblast functions remains to be established.

Our results and those of Antoni et al. (31) show that IL-1β peptide 163-171 is much less potent on a weight and molar basis than hrIL-1β. The reason for the reduced potency of this

IL-1-peptide is unknown. Peptides generally are less potent than intact proteins in binding to receptors and triggering biologic effects of cells as has been demonstrated with growth hormone (43). The reduced potency of the IL-1β peptide may relate to its small size and lack of other sequences necessary for optimal binding to the IL-1 receptor. We plan to explore this possibility in the future by determining the relative effectiveness of this peptide in competing with IL-1β for binding to fibroblast cell surface receptors.

A variety of substances (e.g., phagocytized particles, antigen or mitogen-stimulated lymphocytes, lymphokines, microbial-derived factors, and antigen-antibody complexes) have been reported to stimulate the release of IL-1 from macrophages in vitro (44). These findings would suggest that macrophages present at sites in vivo of inflammatory reactions of diverse etiologies might release IL-1. IL-1 could stimulate neighboring connective tissue fibroblasts to produce increased quantities of HA. It has been demonstrated previously that HA synthesis is accelerated soon after injury to connective tissue (1, 7). The local accumulation of HA could function to facilitate the migration of cells into the reaction site (1–6). Cell migration is effected by the sequential attachment and detachment of cell processes to the substratum. Hyaluronate is thought to facilitate cell migration by virtue of its ability to be interposed between the cell and the substratum, thereby promoting detachment (45, 46). Furthermore, hyaluronate is able to retain large amounts of water within its molecular domain and exerts osmotic pressure on surrounding cells and matrix constituents, thereby opening up new avenues for cell migration (13).

In addition, HA may function as a modulator of immune responses by interfering with binding of antigen or mediators to cell surface receptors and by altering cell–cell interactions (47, 48). In this regard, exogenously added HA has been shown to inhibit mitogen-induced proliferation of lymphocytes, lymphocyte cytotoxicity, and graft-vs.-host reactions (47, 48). Perhaps HA synthesized by fibroblasts after stimulation by IL-1 could act directly or indirectly as a feedback inhibitor of further release of IL-1 from monocytes/macrophages through its ability to modulate immune responses or the interaction of monocytes/macrophages with agents that signal these cells to synthesize and secrete IL-1. The final expression of the biological activity of IL-1 in vivo is no doubt the result of a complex series of events potentially involving other cytokines and specific IL-1 inhibitors (49–51).

This study conclusively demonstrates that human IL-1α and β are capable of stimulating fibroblasts to synthesize HA. This HA may in turn play a pivotal role during the course of immune and inflammatory reactions and the reparative phase that follows such injury to connective tissue by modulating cell–cell interactions.

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