Stimulation of Collagen and Glycosaminoglycan Production in Cultured Human Adult Dermal Fibroblasts by Recombinant Human Interleukin 6

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Interleukin (IL) 6 is a pleiotropic cytokine synthesized by fibroblasts in response to many stimuli, including IL-1β. To evaluate the possibility that previously observed stimulation of fibroblast biosynthetic functions by IL-1β may be mediated by autocrine IL-6, we investigated the effect of recombinant human (rh) IL-6 on the connective tissue–related biosynthetic functions of three lines of cultured human adult dermal fibroblasts. We found that rhIL-6 mimicked some of the activities of IL-1β, as 24–96-h treatment of confluent fibroblast cultures with rhIL-6 caused concentration (10 to 1000 ng/ml)-dependent increases in the production of collagen and the glycosaminoglycans (GAG), hyaluronic acid and chondroitin-4/6-sulfates, but had little effect on fibronectin or total protein production. Although the effective stimulating concentrations of IL-6 were within the range ($\approx$100 ng/ml) we found produced by rhIL-1β–treated fibroblast cultures, rhIL-1β at 0.2–1.0 ng/ml induced significantly greater amounts of collagen and GAG than the maximum effective concentrations of IL-6. Moreover, an anti–rhIL-6 antibody, which effectively neutralized the fibroblast-stimulating activities of rhIL-6, only fractionally blocked the fibroblast-stimulating actions of rhIL-1β, suggesting autocrine IL-6 only partially mediates the effects of IL-1β on fibroblasts. Conversely, the fibroblast-stimulating effects of rhIL-6 are unlikely due to autocrine IL-1β, as an anti–rhIL-1β antibody had only minimal inhibitory action on rhIL-6–treated fibroblast cultures. Overall these results suggest that IL-6 could function as a paracrine/autocrine regulator of dermal fibrotic repair. *J Invest Dermatol 97:686–692, 1991*

Regulation of the fibrotic repair of injured dermal connective tissue appears to be dependent on the ordered growth and differentiation of dermal fibroblasts in response to the orchestrated release of paracrine cytokines by infiltrating platelets, neutrophils, monocytes, T lymphocytes, and other inflammation-associated cells [1,2]. To date, numerous studies suggest that fibroblast-stimulating cytokines such as transforming growth factor-α and -β [2,3], platelet-derived growth factor [2,4], epidermal growth factor [2,5], interleukin (IL) 1α and β, and tumor necrosis factor (TNF) α and -β [6,7] likely control both the connective tissue formation and remodeling phases of dermal fibrotic repair. Moreover, fibroblasts may play a role in regulating their own growth and differentiation as several paracrine cytokines induce fibroblasts to produce additional cytokines that have potential as autocrine regulators of fibroblast functions [6,8–12].

One potential fibroblast autocrine regulator is the increasing important cytokine IL-6, which is synthesized by fibroblast cultures in response to many stimuli including cytokines IL-1β, TNFα, and platelet-derived growth factor [8,9]. IL-6 is a pleiotropic cytokine secreted by several inflammation-associated cell types in addition to fibroblasts [13–18], which interacts with a diverse number of target cell types resulting in a variety of biologic activities including stimulation of the proliferation of hematopoietic stem cells and megakaryocytes, stimulation of hybridoma growth, induction of B-cell differentiation, and stimulation of hepatocytes to produce acute-phase proteins [13,14,19–22]. Moreover, IL-6 has recently been reported to stimulate the synthesis of complement proteins, factor B and C3, in cultured human skin fibroblasts, indicating that fibroblasts are a target cell for paracrine IL-6 and suggesting that previously observed stimulation of fibroblast biosynthetic functions by IL-1β may be mediated by autocrine IL-6 [23].

In order to determine if in addition to complement factors, IL-6 also modulates the connective tissue matrix metabolism of fibroblasts, we measured the production of collagen, glycosaminoglycan (GAG), fibronectin, and total protein by confluent dermal fibroblast microcultures exposed to 10–1000 ng/ml of recombinant human (rh) IL-6 for 24–96 h. Our results demonstrate that rhIL-6 mimics the action of IL-1β by stimulating the production of collagen and GAG (hyaluronic acid and chondroitin-4/6-sulfates) and suggest that paracrine IL-6 could play a role in matrix formation during dermal fibrosis. However, the fibroblast-stimulating effects of IL-1β are likely only partially mediated by autocrine IL-6, as an anti-

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Abbreviations:

DMEM: Dulbecco's modified Eagle's medium
dpm: disintegrations per minute
FCS: fetal calf serum (heat inactivated)
GAG: glycosaminoglycan
IL: interleukin
rh: recombinant human
TCA: trichloroacetic acid
TNF: tumor necrosis factor

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rhIL-6 antibody only fractionally neutralized the stimulating effects of IL-1β on fibroblast cultures.

MATERIALS AND METHODS

Cytokines and Anti-Cytokines Carrier-free rhIL-6 and rhIL-1β derived from *Escherichia coli* were obtained from R & D Systems, Minneapolis, MN. The manufacturer reported the biologic activity of rhIL-6 to average 1.0×10^6 U/mg in the plasmacytoma growth factor assay using T1165.85.2.1 cells, and we found the rhIL-6 to have an average activity of 5.0×10^8 U/mg is the hybridoma growth factor assay using B-9 cells [20]. RhIL-1β was reported by the manufacturer to have an activity of 5×10^8 U/mg in the thymocyte proliferation assay. Carrier-free, *E. coli*–derived rhTNFα, with a cytotoxic activity of 5.0×10^7 U/mg, was a gift from Genentech Inc., South San Francisco, CA. Lipopolysaccharide (*E. coli*; 0128:B12) was obtained from Sigma Chemical, St. Louis, MO. All cytokine preparations contained endotoxin levels less than 0.1 ng per 1.0 μg of cytokine and all tissue culture procedures used in this study were performed with media and reagents judged to contain less than 0.02 ng/ml endotoxin by the Limulus amebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD). Such precautions prevent enhanced constitutive synthesis of IL-6 by endotoxin-stimulated fibroblast microcultures.

An anti–rhIL-6 antibody raised in goats was obtained from R&D Systems and an anti–rhIL-1β murine monoclonal antibody was obtained from Cistron Biotechnology, Pine Brook, NJ. Both antibodies were supplied in phosphate-buffered saline and 1.0 ml of anti–IL-6 neutralized 20,000 ng of rhIL-6 in the plasmacytoma growth factor assay and 1.0 ml of anti–IL-1β neutralized 5 ng of rhIL-1β in the thymocyte proliferation assay. Cytokines and anti-cytokines were diluted to desired concentrations with Dulbecco's modified Eagle's medium (DMEM) containing 0.1% human serum albumin and either added directly to fibroblast cultures, or in the case of antibody neutralization studies preincubated for 1 h at 37°C before addition to cultures. In all experiments control cultures were treated with appropriate volumes of DMEM containing 0.1% albumin.

Fibroblast Cultures The normal adult dermal fibroblast lines used in this study were previously established using the explant method from samples of skin removed during cosmetic surgery. Line 1 was derived from the mammary skin of a 35-year-old woman, line 2 from the facial skin of a 52-year-old man, and line 3 from the facial skin of a 42-year-old woman. The fibroblasts were maintained in DMEM containing 25 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin plus 10% heat-inactivated fetal calf serum (FCS) (Whittaker Bioproducts) at 37°C in a 5% CO_2 humidified atmosphere. Fibroblast cultures were subcultured by trypsinization and fibroblasts from the third to the tenth passages were then assayed for biosynthetic activities using confluent cultures to minimize any cytokine-induced effects on cell growth. We have previously reported on the effects of IL-1α and β and TNFα and β on these fibroblast lines [7].

Assay of Fibroblast Collagen Production Assay of collagen production during a 24-h period by confluent fibroblasts cultured in the presence of ascorbic acid and 0, 1, or 10% FCS was assessed by ^{3}H-proline incorporation into collagenous protein. Freshly trypsinized fibroblasts were plated in 96-well microcultures (0.45 cm^2 surface area) at a near-confluent density of 25,000 fibroblasts per well in 200 μl DMEM-10% FCS and incubated for 48 h to produce a totally confluent monolayer of fibroblasts. The medium was then removed and replaced with 200 μl DMEM without glutamine containing 0, 1, or 10% FCS and 50 μg/ml ascorbic acid with or without cytokines or anti-cytokines and incubated for an additional 24–96 h. Cultures were pulsed with 0.5 μCi of L-[5-^{3}H]proline (31 Ci/mmol; Amersham Corp, Arlington Heights, IL) and 50 μg/ml beta-aminopropionitrile and fresh ascorbic acid for the final 24 h of culture. The ^{3}H-proline incorporation into pepsin-resistant, salt-precipitated extracellular collagen was then determined as previously described and averaged results for triplicate microcultures are expressed as disintegrations per minutes (dpm) of ^{3}H-collagen per 10^3 cells ± SD to account for any possible cytokine-induced differences in cell numbers [7,24]. Harvested microcultures contained from 35,000 to 65,000 fibroblasts, dependent upon length of incubation, culture conditions, and cytokine treatment. Harvested fibroblasts were always greater than 95% viable (trypan blue exclusion) regardless of culture conditions.

Assay of Fibroblast GAG Production Microcultures treated identically to those described above for assay of collagen production were used to assess GAG production except they were incubated without beta-aminopropionitrile and labeled with 0.5 μCi of D-[6-^{3}H]glucosamine hydrochloride (22 Ci/mmol, Amersham) for the terminal 24 h of culture. Following treatment with pronase, the ^{3}H-glucosamine incorporation into cetylpyridinium chloride precipitated material in the combined culture media and cell layer was then determined as previously described and averaged results for triplicate microcultures are expressed as dpm of ^{3}H-GAG per 10^3 cells ± SD [7,25].

Analysis of individual ^{3}H-GAG types produced was performed using the differential GAGase method [26]. After digestion of the cell layer with 0.5 N NaOH and neutralization, microcultures containing 250 μl of combined culture media and digested cell layer were treated with GAGases (Sigma Chemical Co., St. Louis, MO) as follows. (a) Fungal hyaluronidase (*Streptomyces hyaluronlyticus*): 1.0 U in 25 μl of 0.2 M acetate buffer, ph 5.0, for 20 h at 55°C. (b) Chondroitinase AC: 0.1 U in 25 μl of 0.8 M Tris buffer, ph 8.0, for 20 h at 37°C. (c) Chondroitinase ABC: 0.1 U in 25 μl of 0.8 M Tris buffer, ph 8.0, for 20 h at 37°C. Following treatment with pronase, ^{3}H-GAG were precipitated and quantitated as described above. Subtraction of labeled GAG resistant to hyaluronidase from total precipitated GAG radioactivity determined the quantity of hyaluronic acid. Subtraction of ^{3}H-labeled GAG resistant to chondroitinase AC from the hyaluronidase resistant counts determined the quantity of chondroitin-4/6-sulfates. Similarly, the quantity of dermatan sulfate was determined by subtracting the chondroitinase ABC resistant counts from chondroitinase AC resistant counts. Other GAG are the counts not digested by chondroitinase ABC.

Assay of Fibroblast Fibronectin Production Microcultures identical to those described above were used to assess fibronectin production except they were pulsed with only fresh ascorbic acid for the terminal 24 h of culture. After 24–96 h incubation, supernatant medium was removed and the fibronectin content of the medium was determined using a commercially available immunoturbidimetric assay kit (Boehringer-Mannheim, Indianapolis, IN) [7,25]. This assay uses an antisera to human fibronectin that has minimal cross-reactivity with bovine fibronectin, allowing the accurate assay of the fibronectin content of culture supernatants containing 10% FCS after subtraction of a small background value. Each experimental condition was performed in quintuplicate wells and averaged results obtained by measuring the fibronectin concentration of 0.3 ml aliquots of pooled supernatant medium are reported as ng per 10^3 cells ± SD.

Assay of Fibroblast Total Protein Production Confluent microcultures prepared identically to those described above for assay of collagen production were used to assess ^{3}H-tryptophan incorporation into newly produced protein. However, cytokine treatments were performed for 72 h in Ham's F-10 medium (lower tryptophan content than DMEM) + 1% FCS and without ascorbic acid. Cultures were pulsed with 0.5 μCi of L-[5-^{3}H]tryptophan (31.5 Ci/mmol; NEN, Boston, MA) for the final 24 h of culture. ^{3}H-tryptophan labeled proteins in the cell layer and supernatant media were separated and precipitated at 4°C with 10% trichlorocetic acid (TCA) and collected on glass fiber filters as previously described [24]. Averaged results for triplicate microcultures are expressed as dpm of ^{3}H-protein per 10^3 cells ± SD.

Assay of Fibroblast IL-6 Production The IL-6 content of supernatant media from fibroblast microcultures was measured as hybridoma growth-factor activity using the IL-6–dependent murine

B-cell hybridoma, B9, as previously described [20]. Briefly, microcultures of 2000 B9 cells in 200 μl of RPMI-1640 containing 10% FCS and 10^{-4} M 2-mercaptoethanol were cultured for 72–96 h with different dilutions of exhaustively dialyzed fibroblast supernatants or appropriate concentrations of rhIL-6 standards. Assays were performed in triplicate and microcultures were pulsed with 0.5 μCi/well of [methyl-^{3}H]thymidine (82 Ci/mmol; NEN) for the terminal 4 h of culture before harvesting onto glass fiber filters using an automated harvester. Supernatant IL-6 concentrations were calculated from concentration-response curves obtained with rhIL-6, which had an average ED_{50} (concentration producing half-maximal response) of 2.0 pg/ml.

Statistical Analysis Differences between mean values were assessed for statistical significance ($p < 0.05$) by the two-tailed Student t test.

RESULTS

Fibroblast IL-6 Production In order to determine the appropriate concentrates of rhIL-6 to test for effects on fibroblast functions we assayed the constitutive and induced production of IL-6 by dermal fibroblast line 1 over a 48-h period in DMEM + 1% FCS. Fibroblast microcultures were either left untreated or stimulated with 1 ng/ml rhIL-1β, 200 ng/ml rhTNFα, or 10 μg/ml lipopolysaccharide. The IL-6 concentrations produced were untreated control, 1.2 ± 0.1 ng/ml; IL-1β stimulated, 118.3 ± 10.2 ng/ml; TNFα stimulated, 71.6 ± 8.5 ng/ml; and lipopolysaccharide stimulated, 37.5 ± 2.5 ng/ml. The constitutive IL-6 production of several other fibroblast lines, including lines 2 and 3, were also assayed and fell in the range 0.5 to 8.0 ng/ml whether incubation was performed in DMEM alone or DMEM + 1% or 10% FCS. These results agree with the previous reports that fibroblast cultures constitutively produce 0.2 to 3.0 ng/ml of hybridoma growth factor assayable IL-6, and that IL-1 and TNF-stimulation increases IL-6 production by up to 200 times [9,27]. IL-6 concentrations in the cytokine-induced range (10–1000 ng/ml) were thus chosen for subsequent experiments modulating fibroblast biosynthetic functions.

Effect of IL-6 on Fibroblast Collagen Production We have previously reported that IL-1α and β effectively induce the collagen production of dermal fibroblasts cultured in DMEM containing no FCS, but that IL-1 has little stimulatory or even inhibitory effects on collagen production in 10% FCS [7]. Subsequent experiments have demonstrated that IL-1β maximally stimulates fibroblast collagen production in 1% FCS rather than in DMEM alone (data not shown). Although the reasons for more effective stimulation of collagen production in low concentrations of FCS are uncertain, similar observations have been made by others and may be dependent on autocrine prostaglandin production [28–30]. Therefore, we studied the effect of rhIL-6 on the collagen production of confluent fibroblast microcultures incubated in DMEM alone, or DMEM + 1% or 10% FCS. Preliminary studies incubating line 1 fibroblasts with 100 ng/ml of rhIL-6 for 24–96 h in DMEM + 1% FCS established that rhIL-6 optimally stimulates collagen production after 72 h (data not shown). As detailed in Table I, 72-h treatment with rhIL-6 stimulated the collagen production of fibroblast line 1 most effectively in DMEM + 1% FCS, causing statistically significant increases of 27, 51, and 76% at 100, 300, and 1000 ng/ml of rhIL-6. IL-6 was ineffective in DMEM + 10% FCS and only the highest IL-6 concentration (1000 ng/ml) caused a statistically significant stimulation of collagen production in DMEM alone.

To ensure that stimulation of collagen production by rhIL-6 was not restricted to one fibroblast line, two additional adult dermal fibroblast lines cultured in DMEM + 1% FCS were treated with rhIL-6 for 72 h. As shown in Table II, fibroblast lines 2 and 3 responded similarly to fibroblast line 1, with concentrations of rhIL-6 greater than 100 or 300 ng/ml causing statistically significant increases in collagen production. Because the three fibroblast lines tested were derived from skin taken from different anatomic sites (breast, face) of both males and females, the data suggest that rhIL-6 will similarly affect the collagen production of most adult dermal fibroblast lines.

Table I. Effect of rhIL-6 on Collagen Production by Dermal Fibroblast Line 1[a]

IL-6 (ng/ml)	^{3}H-Collagen DPM/10^3 Cells ± SD		
	DMEM	DMEM + 1% FCS	DMEM + 10% FCS
0	45.2 ± 0.6	71.5 ± 5.6	107.3 ± 11.4
10	44.1 ± 5.2	75.8 ± 4.5	118.1 ± 7.6
30	43.0 ± 5.6	77.0 ± 5.4	121.3 ± 7.3
100	45.4 ± 2.7	90.6 ± 7.6	104.1 ± 9.6
300	43.9 ± 1.9	108.0 ± 5.6	111.9 ± 9.4
1000	62.2 ± 8.1	125.8 ± 2.4	110.5 ± 6.8

[a] Collagen production by fibroblast microcultures during the terminal 24 h of a 72-h treatment with rhIl-6 was assessed by ^{3}H-proline incorporation into pepsin-resistant, salt-precipitated collagenous protein as described in *Materials and Methods.* Data were confirmed in two additional experiments.

Effect of IL6 on Fibroblast GAG Production As we and others have previously reported that IL-1α and β are potent stimulators of fibroblast GAG synthesis we next examined the effect of rhIL-6 on adult dermal fibroblast GAG production [7,26]. A preliminary kinetic study, incubating line 1 fibroblasts with 100 ng/ml of rhIL-6 in DMEM alone or DMEM + 10% FCS indicated that rhIL-6 optimally stimulates GAG production after 72 h treatment (data not shown). As detailed in Table III, 72-h treatment with rhIL-6 stimulated the production of GAG by fibroblast line 1 equally well in the absence or presence of 10% FCS, causing statistically significant increases of 21–97% at 100–1,000 ng/ml of rhIL-6. As shown in Table II, rhIL-6 also increased the production of GAG by fibroblast lines 2 and 3 cultured in DMEM, with 300 and 1000 ng/ml of rhIL-6 inducing statistically significant enhancement of GAG production by 20 and 85%, respectively.

An analysis of individual GAG types produced by fibroblast line 1 in DMEM + 10% FCS was done using the differential GAGase digestion method [26]. Hyaluronic acid was the major GAG species produced by control cultures, as 70.4% of the induced ^{3}H-GAG was digested by hyaluronidase (Table IV). Chondroitin-4/6-sulfates accounted for 5.1% of the ^{3}H-GAG in control cultures, whereas dermatan sulfate accounted for 17.8% and other GAG a residual 6.7%. Treatment with 1000 ng/ml of IL-6 induced statistically significant increases in the production of hyaluronic acid (157%) and chondroitin-4/6-sulfates (60%). IL-6 treatment had no significant effect on the production of dermatan sulfate or other residual GAG.

Table II. Effect of rhIL-6 on Biosynthetic Functions of Dermal Fibroblast Lines 2 and 3[a]

Fibroblast Production	IL-6 (ng/ml)	Fibroblast Line 2	Fibroblast Line 3
Collagen (DPM/10^3 cells ± SD)	0	156.1 ± 14.6	124.9 ± 7.6
	100	171.8 ± 21.2	234.5 ± 25.3
	300	201.8 ± 11.6	250.5 ± 21.4
	1000	219.5 ± 14.1	457.6 ± 53.1
GAG (DPM/10^3 cells ± SD)	0	214.4 ± 24.0	77.7 ± 9.1
	100	249.3 ± 2.8	92.1 ± 7.4
	300	293.2 ± 27.7	92.9 ± 7.8
	1000	396.7 ± 40.9	105.1 ± 5.6
Fibronectin (ng/10^3 cells ± SD)	0	18.3 ± 1.0	12.6 ± 1.3
	100	19.0 ± 1.2	12.8 ± 0.7
	300	19.5 ± 0.9	11.4 ± 1.1
	1000	19.5 ± 0.8	10.2 ± 1.4

[a] Fibroblast microculture conditions and assays of biosynthetic products are as described in Tables I and III, except collagen production was assayed in only DMEM + 1% FCS and GAG and fibronectin production in only DMEM alone. Data were confirmed in one additional experiment.

Table III. Effect of rhIL-6 on Glycosaminoglycan and Fibronectin Production by Dermal Fibroblast Line 1[a]

IL-6 (ng/ml)	^{3}H-GAG DPM/10^3 Cells ± SD		Fibronectin ng/10^3 Cells ± SD	
	DMEM	DMEM + 10% FCS	DMEM	DMEM + 10% FCS
0	95.8 ± 4.0	210.4 ± 33.5	15.3 ± 0.8	26.4 ± 2.2
10	96.0 ± 11.7	221.5 ± 5.1	15.0 ± 1.0	27.0 ± 2.5
30	87.4 ± 8.9	246.5 ± 11.2	16.3 ± 1.2	25.0 ± 1.8
100	115.6 ± 8.2	320.7 ± 49.2	16.3 ± 0.6	26.0 ± 0.9
300	132.0 ± 14.8	343.4 ± 59.2	16.1 ± 1.6	26.5 ± 2.7
1000	161.5 ± 2.4	415.1 ± 67.0	17.7 ± 1.5	27.0 ± 2.2

[a] GAG production by fibroblast microcultures during the terminal 24 h of a 72-h treatment with rhIL-6 was assessed by ^{3}H-glucosamine incorporation into cetylpyridinium chloride precipitated material as described in *Materials and Methods*. Fibronectin production during a 72-h period was assessed by an immunoturbidimetric assay of fibroblast culture media as described in *Materials and Methods*. Data was confirmed in three additional experiments.

Treatment with 1 ng/ml of rhIL-1β also preferentially stimulated hyaluronic acid production (282%) with less enhancement of chondroitin-4/6-sulfates (111%), as has previously been reported [26].

Effect of IL-6 on Fibroblast Fibronectin Production Because fibronectin is another connective tissue matrix component whose synthesis by fibroblasts is modulated by IL-6–inducing cytokines, such as IL-1α and β [7,29,31], we studied the effect of rhIL-6 on adult dermal fibroblast fibronectin production. Although our three fibroblast lines produced 12.6–26.4 ng of fibronectin per 10^3 cells during 72 h of incubation, concentrations of rhIL-6 up to 1000 ng/ml had no significant effect on fibronectin production after 24–96 h of treatment either in the absence or presence of 10% FCS (Tables II and III). This contrasts with IL-1β, which decreases fibroblast fibronectin production [7,29,31].

Effect of IL-6 on Fibroblast Total Protein Production In order to determine if the IL-6 enhancement of collagen and GAG production is specific for these extracellular matrix structural molecules or the result of a general stimulatory effect upon most cellular functions, we investigated the effect of rhIL-6 on the incorporation of ^{3}H-tryptophan into intracellular and secreted protein. ^{3}H-tryptophan incorporation into TCA-precipitable material is a preferential assay for total noncollagenous protein synthesis, as tryptophan is absent in the helical portions of collagen types I and III alpha chains and present in only 4/1000 residues in procollagen I and III, whereas other proteins generally have higher tryptophan contents, e.g., fibronectin, 36/1000 residues [32,33]. The assays were performed in media without ascorbic acid to further favor noncollagenous protein production, but over a 72-h period in 1% FCS, conditions that allow for maximal IL-6 stimulation of collagen production. Under these conditions rhIL-6, at concentrations up to 1000 ng/ml, failed to induce significant increases in the secreted protein production of fibroblast lines 1, 2, and 3, or in the cellular protein of lines 1 and 2 (Table V). 1000 ng/ml of rhIL-6 did, however, cause a statistically significant increase of 32% in the cellular protein production of fibroblast line 3. Interestingly, fibroblast line 3 is the most sensitive to the stimulating effect of IL-6 on collagen production, suggesting that the measured increase in cellular protein could be accounted for by 3H-tryptophan incorporation into procollagens. The lack of effect of IL-6 on total protein and fibronectin production suggests that the stimulating effects of IL-6 on fibroblast functions are relatively specific for collagen and GAG.

Effect of Anti–IL-6 on IL-6 and IL-1–Induced Fibroblast Functions Although the rhIL-6 preparation tested was greater than 95% pure and contained negligible endotoxin, we confirmed that IL-6 was the molecule in the IL-6 preparation acting on fibroblasts by performing neutralization studies with an anti–rhIL-6 antibody. As shown in Table VI, anti–IL-6 antibody at 12.5 μl/ml reduced rhIL-6 stimulation by statistically significant amounts, almost completely blocking the stimulation of collagen and GAG production, induced by 250 ng/ml of rhIL-6. As would be expected, addition of anti–IL-6 antibody to control cultures had no effect on the production of collagen or GAG as constitutive production of IL-6 by such cultures (0.5–8.0 ng/ml) is less than the minimum effective concentration of rhIL-6 (data not shown). Because our results show that rhIL-6 mimics the activities of IL-1β on fibroblasts, by stimulating collagen and GAG production, suggesting that autocrine IL-6 may mediate the action of IL-1β, we directly compared the effectiveness of rhIL-6 and rhIL-1β and investigated if anti–IL-6 could neutralize the action of IL-1β. As shown in Table VI, as little as 1.0 ng/ml of rhIL-1β was 3 times as effective in stimulating collagen and GAG production as was 250 ng/ml of rhIL-6. However, anti–IL-6 did partially block the stimulatory actions of IL-1β on GAG production by statistically significant amounts, but was ineffective on collagen production, as detailed in Table VI. These results indicate that rhIL-6 is a much weaker mediator of fibroblasts functions than is rhIL-1β and that autocrine IL-6 at the concentrations produced only partially mediates the effects of IL-1β on fibroblasts.

Effect of Anti–IL-1 on IL-6 and IL-1–Induced Fibroblast Functions Although fibroblasts do not constitutively produce IL-1β, at least one cytokine, namely TNFα, has been reported to induce fibroblasts to produce IL-1β [6]. Therefore, we investigated if an anti–rhIL-1β antibody could block the stimulatory effects of rhIL-6 on fibroblasts to ensure that the activities of IL-6 were not due to rhIL-6 induction of autocrine IL-1β. The results detailed in

Table IV. Effects of rhIL-6 and rhIL-1β on the Profile of Glycosaminoglycans Produced by Dermal Fibroblast Line 1[a]

Treatment	^{3}H-Glycosaminoglycan DPM/10^3 Cells ± SD			
	Hyaluronic Acid	Chondroitin –4/6 Sulfates	Dermatan Sulfate	Other GAG
None	123.3 ± 11.2	8.8 ± 1.2	31.2 ± 2.8	11.9 ± 1.2
IL-6 (1000 ng/ml)	317.3 ± 25.1	14.1 ± 1.3	29.7 ± 3.2	11.8 ± 0.9
IL-1 (1 ng/ml)	477.0 ± 49.5	18.6 ± 1.5	29.8 ± 3.5	12.2 ± 1.0

[a] GAG production by fibroblast microcultures during the terminal 24 h of a 72-h treatment with rhIL-6 or rhIL-1β was assessed by ^{3}H-glucosamine incorporation into cetylpyridinium chloride-precipitated material following digestion with hyaluronidase, chondroitinase AC, and chondroitinase ABC as described in *Materials and Methods*.

Table V. Effect of rhIL-6 on Total Protein Production by Dermal Fibroblasts[a]

IL-6 (ng/ml)	^{3}H-Tryptophan-Labeled Protein DPM/10^3 Cells ± SD: Fibroblast Line 1 Media	Fibroblast Line 1 Cell	Fibroblast Line 2 Media	Fibroblast Line 2 Cell	Fibroblast Line 3 Media	Fibroblast Line 3 Cell
0	127.2 ± 12.9	798.4 ± 94.0	174.1 ± 18.3	670.2 ± 79.3	198.8 ± 8.7	522.0 ± 39.2
100	119.4 ± 19.1	670.9 ± 33.8	179.3 ± 23.8	665.8 ± 69.5	198.4 ± 41.7	584.1 ± 79.2
300	138.2 ± 20.8	817.2 ± 77.3	156.6 ± 21.5	678.9 ± 67.3	206.2 ± 15.7	576.4 ± 5.7
1000	110.1 ± 5.3	724.7 ± 57.0	184.7 ± 11.2	701.3 ± 37.9	212.2 ± 25.1	687.1 ± 19.7

[a] Protein production by fibroblast microcultures during the terminal 24 h of a 72-h treatment with rhIL-6 in Ham's F-10 medium + 1% FCS was assessed by ^{3}H-tryptophan incorporation into TCA-precipitated material in the culture media and cell layer as described in *Materials and Methods.*

Table VII again show that rhIL-1β is a much more effective stimulator of fibroblast functions than is rhIL-6 and that 60 μl/ml of anti-IL-1 β antibody reduced rhIL-1β stimulations by statistically significant amounts, almost completely neutralizing the effects of 0.2 ng/ml of IL-1β. Anti-IL-1β had no significant effect on IL-6-induced GAG production but did partially inhibit collagen production, as detailed in Table VII. However, the effect on collagen production is likely nonspecific as anti-IL-1β also depressed the collagen production of control cultures by approximately 25% but had no effect on GAG production (data not shown). Ascites-derived monoclonal antibodies have been reported to be contaminated with cytokines, which could be responsible for the nonspecific inhibition of collagen production observed [34]. These results suggest that the stimulatory effects of rhIL-6 on fibroblast functions are not mediated by autocrine IL-1β.

DISCUSSION

Recent studies showing dermal fibroblasts to be target cells for IL-6 [23] and that several wound healing-associated cell types (neutrophils, monocytes, T lymphocytes, vascular endothelial and smooth muscle cells, keratinocytes, and fibroblasts) secrete IL-6 in vitro suggest that IL-6 could function as a paracrine and/or autocrine regulator of dermal fibrotic repair [8,9,13-18,35-37]. The purpose of this study was, then, to determine if IL-6 plays a role in dermal connective tissue metabolism by investigating the effect of rhIL-6 on the biosynthetic functions of cultured adult dermal fibroblasts. Our study demonstrates that rhIL-6 stimulates dermal fibroblasts to produce increased amounts of two connective-tissue matrix components, collagen and GAG, without increasing fibronectin or general protein synthesis. These results suggest that paracrine IL-6 could play a role in the matrix formation phase of dermal fibrotic repair by inducing the synthesis of new collagen and GAG.

Studies investigating the effects of rhIL-6 on the connective tissue matrix metabolism of dermal fibroblasts have previously been reported by ourselves and others in abstract form [38,39]. Our previous study performed with rhIL-6 from a different source than the rhIL-6 used in this paper, also showed that rhIL-6 stimulates GAG production. However, the samples of rhIL-6 (Genzyme, Boston, MA) we used in our abstracted report inhibited collagen and fibronectin production. We have subsequently found these two effects not to be neutralized by anti-IL-6 antibody and speculate they could be due to carrier bovine serum albumin, added to the rhIL-6 preparation by the manufacturer, being contaminated with serum-derived cytokines. To negate such a complication the present study was performed with rhIL-6 supplied carrier-free and diluted in our laboratory with human serum albumin prescreened for negative effects on assayed fibroblast functions. The other abstracted report demonstrated that IL-6 stimulates fibroblast production of collagenase mRNA and collagenase protein detectable by anti-collagenase antibodies [39]. Analysis of fibroblast supernatants generated in our current study show that 1000 ng/ml of rhIL-6 increases fibroblast production of trypsin-activatable collagenolytic activity by amounts of 56-130%, when assayed by solubilization of ^{14}C-labeled collagen gels (data not shown). These combined preliminary observations suggest that IL-6 may also play a role in matrix remodeling during dermal fibrotic repair.

Because cultured fibroblasts synthesize IL-6 in basal amounts constitutively, and in enhanced amounts after treatment with cytokines whose activity mimics rhIL-6, such as IL-1α/β and TNFα/β [8,9,27], we investigated if autocrine IL-6 could contribute to regulating dermal fibrotic repair. We found the constitutive levels of IL-6 produced by fibroblast microcultures (0.5 to 8.0 ng/ml) to be below the minimum effective concentrations of rhIL-6 that affected fibroblast biosynthetic functions (30-100 ng/ml). However, stimulation with IL-1β or TNFα increased the IL-6 concentration of fibroblast supernatant media to approximately 100 times constitutive levels, well within the effective concentration range of rhIL-6 on fibroblast biosynthetic functions. However, fibroblast microcultures treated with 0.2-1.0 ng/ml of rhIL-1β produced markedly greater amounts of collagen and GAG than the maximum tested concentration of 1000 ng/ml of rhIL-6, suggesting autocrine IL-6

Table VI. Effect of Anti-rhIL-6 on Dermal Fibroblast Biosynthetic Functions Induced by rhIL-6 and rhIL-1β[a]

Fibroblast Treatment	^{3}H-Collagen Production DPM/10^3 Cells ± SD	^{3}H-GAG Production DPM/10^3 Cells ± SD
None	131.7 ± 7.7	49.2 ± 5.4
IL-6 (250 ng/ml)	192.0 ± 13.0	72.7 ± 3.5
IL-6 + Anti-IL-6	127.7 ± 6.4	53.1 ± 4.1
IL-1 (1.0 ng/ml)	319.0 ± 30.6	117.3 ± 5.0
IL-1 + Anti-IL-6	323.4 ± 19.2	99.6 ± 8.8

[a] Fibroblast microculture conditions and assays of biosynthetic products are as described in Table II. Collagen production was assessed on fibroblast line 3, and GAG production on fibroblast line 1. Anti-IL-6 was used at a concentration of 12.5 μl/ml, a quantity sufficient to neutralize 250 ng/ml of rhIL-6 in the plasmacytoma growth factor assay. Data were confirmed in two additional experiments.

Table VII. Effect of Anti-rhIL-β on Dermal Fibroblast Biosynthetic Functions Induced by rhIL-6 and rhIL-1β[a]

Fibroblast Treatment	^{3}H-Collagen Production DPM/10^3 Cells ± SD	^{3}H-GAG Production DPM/10^3 Cells ± SD
None	172.6 ± 10.3	73.9 ± 10.2
IL-6 (500 ng/ml)	360.6 ± 10.8	122.2 ± 10.9
IL-6 + Anti-IL-1	260.1 ± 27.9	112.3 ± 6.6
IL-1 (0.2 ng/ml)	408.9 ± 25.2	194.4 ± 15.7
IL-1 + Anti-IL-1	216.3 ± 21.1	87.3 ± 9.6

[a] Microculture and assay conditions are as described in Table VI. Anti-IL-1β was used at a concentration of 60 μl/ml, a quantity sufficient to neutralize 0.3 ng/ml of rhIL-1β in the thymocyte proliferation assay. Data were confirmed in one additional experiment.

could not be entirely responsible for the fibroblast-stimulatory effects of rhIL-1β. Moreover, an anti–rhIL-6 antibody only fractionally blocked the fibroblast-stimulatory actions of rhIL-1β, verifying that autocrine IL-6 likely only partially mediates the effects of IL-1β on fibroblasts. Overall these results suggest that cytokine stimulation produces autocrine IL-6 in sufficient quantities to affect in situ dermal repair, but that at least in the case of IL-1β, the fibroblast-stimulatory effects of the IL-6–inducing cytokine overshadow the effects of autocrine IL-6.

We found that a concentration range of 100–1000 ng/ml of rhIL-6 was necessary to effectively stimulate fibroblast collagen and GAG production. Whereas these concentrations are approximately 10–100 times greater than those required to stimulate other IL-6 target cells [19–22], they are similar to the concentrations of rhIL-6 previously reported to induce complement factor production by fibroblasts [23]. It seems likely that concentrations of 100–1000 ng/ml of IL-6 could be achieved in situ during dermal fibrotic repair as in vitro studies suggest the combined IL-6 production of several wound healing-associated cells would exceed 1000 ng/ml. For example, following in vitro stimulation monocytes had been reported to produce up to 100 ng/ml of IL-6; vascular endothelial cells, 50 ng/ml; vascular smooth muscle cells, 1000 ng/ml; and fibroblasts, 100 ng/ml [9,15,17,18]. Moreover, fluid sampled from arthritic synovium and from cerebrospine of patients with meningococcal meningitis have been reported to contain 100 to 1000 ng/ml of IL-6, further suggesting that other localized inflammatory sites, such as dermal wounds, could contain concentrations of IL-6 high enough to stimulate fibroblast collagen and GAG production [40,41].

Overall, our results support the hypothesis that IL-6 could function as a paracrine/autocrine regulator of normal dermal fibrotic repair, and further suggest that IL-6 could play a role in the pathophysiology of dermal fibrotic diseases such as scleroderma or keloids by inducing the hypercollagen/GAG-producing phenotype characteristic of fibroblasts derived from sites of dermal fibrosis [42,43]. However, our preliminary results indicate that fibroblasts derived from sclerodermal or keloidal skin respond to paracrine rhIL-6 and produce constitutive and IL-1β/TNFα-induced autocrine IL-6 in a manner analagous to normal fibroblasts (data not shown). Thus, paracrine IL-6 could be involved in inducing the fibrotic fibroblast phenotype, but it is unlikely that autocrine IL-6 is responsible for the maintenance of the hypercollagen/GAG-producing phenotype displayed by cultured sclerodermal and keloidal fibroblasts.

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REFERENCES

1. Clark RAF: Cutaneous tissue repair: basic biologic considerations. J Am Acad Dermatol 13:701–725, 1985
2. Sporn MB, Roberts AB: Peptide growth factors and inflammation, tissue repair and cancer. J Clin Invest 78:329–332, 1986
3. Sporn MB, Roberts AB, Wakefield LM, deCrombrugghe B: Some recent advances in the chemistry and biology of transforming growth factor-beta. J Cell Biol 105:1039–1045, 1988
4. Deuel TF, Huang JS: Platelet-derived growth factor: structure, function and roles in normal and transformed cells. J Clin Invest 74:669–676, 1984
5. Laato M, Kahari V-M, Niinikoski J, Vuorio E: Epidermal growth factor increases collagen production in granulation tissue by stimulation of fibroblast proliferation and not activation of procollagen genes. Biochem J 247:385–388, 1987
6. Le J, Vilcek J: Tumor necrosis factor and interleukin 1: Cytokines with multiple overlapping biological activities. Lab Invest 56:234–248, 1987
7. Duncan MR, Berman B: Differential regulation of collagen, glycosaminoglycan, fibronectin and collagenase activity production in cultured human dermal fibroblasts by interleukin 1-alpha and beta and tumor necrosis factor-alpha and beta. J Invest Dermatol 92:699–706, 1989
8. Kohase M, May LT, Tamm I, Vilcek J, Sehgal PB: A cytokine network in human diploid fibroblasts: interactions of β-interferons, tumor necrosis factor, platelet derived growth factor, and interleukin-1. Mol Cell Biol 7:273–280, 1987
9. Van Damme J, Cayphas S, Opdenakker G, Billiau A, Van Snick J: Interleukin 1 and poly(rI).poly(rC) induce production of a hybridoma growth factor by human fibroblasts. Eur J Immunol 17:1–7, 1987
10. Kaushansky K, Lin N, Adamson JW: Interleukin 1 stimulates fibroblasts to synthesize granulocyte-macrophage and granulocyte colony-stimulating factors. J Clin Invest 81:92–97, 1988
11. Raines EW, Dower SK, Ross R: Interleukin 1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA. Science 243:393–396, 1989
12. Matsushima K, Oppenheim JJ: Interleukin 8 and MCAF: novel inflammatory cytokines inducible by IL1 and TNF. Cytokine 1:2–13, 1989
13. Sehgal PB, May LT, Tamm I, Vilcek J: Human β_2 interferon and B-cell differentiation factor BSF-2 are identical. Science 235:731–732, 1987
14. Wong GG, Clark SC: Multiple actions of interleukin 6 within a cytokine network. Immunol Today 9:137–139, 1988
15. Sironi M, Breviario F, Proserpio P, Biondi A, Vecchi A, Van Damme J, Dejana E, Mantovani A: IL-1 stimulates IL-6 production in endothelial cells. J Immunol 142:549–553, 1989
16. Guerne P-A, Carson DA, Lotz M: IL-6 production by human articular chondrocytes. J Immunol 144:499–505, 1990
17. Loppnow H, Libby P: Proliferating or interleukin 1-activated human vascular smooth muscle cell secrete copious interleukin 6. J Clin Invest 85:731–738, 1990
18. Aarden LA, De Groot ER, Schaap OL, Landsdorp PM: Production of hybridoma growth factor by human monocytes. Eur J Immunol 17:1411–1416, 1987
19. Ikebuchi K, Wong GG, Clark SC, Ihle JN, Hirai Y, Ogawa M: Interleukin-6 enhancement of interleukin-3 dependent proliferation of multipotential hemopoietic progenitors. Proc Natl Acad Sci USA 84:9035–9039, 1987
20. Van Damme J, Van Beeumen J, Decock B, Van Snick J, De Ley M, Billiau A: Separation and comparison of two monokines with lymphocyte activating factor activity: IL-1β and hybridoma growth factor (HGF): identification of leukocyte-derived HGF as IL-6. J Immunol 140:1534–1541, 1988
21. Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, Kashiwamura S, Nakajima K, Koyama K, Iwamatsu A, Tsunasawa S, Sakiyama F, Matsui H, Takahara Y, Taniguchi T, Kishimoto T: Complementary DNA for a novel human interleukin (BSF-2) that induces B-lymphocytes to produce immunoglobulin. Nature 324:73–76, 1986
22. Gauldie J, Richards C, Harnish D, Landsdorp P, Baumann H: Interferon β_2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. Proc Natl Acad Sci USA 84:7251–7255, 1987
23. Katz Y, Revel M, Strunk RC: Interleukin 6 stimulates synthesis of complement proteins, factor B and C 3 in human skin fibroblasts. Eur J Immunol 19:983–988, 1989
24. Duncan MR, Berman B: γ-interferon is the lymphokine and β-interferon the monokine responsible for inhibition of fibroblast collagen production and late but not early fibroblast proliferation. J Exp Med 162:516–527, 1985
25. Duncan MR, Berman B: Differential regulation of glycosaminoglycan, fibronectin and collagenase production in cultured human dermal fibroblasts by interferons-alpha, beta and gamma. Arch Dermatol Res 281:11–18, 1989
26. Postlethwaite AE, Smith GN Jr, Lachman LB, Endres RO, Poppleton HM, Hasty KA, Seyer JM, Kang AH: Stimulation of glycosaminoglycan synthesis in cultured dermal fibroblasts by interleukin 1. J Clin Invest 83:629–636, 1989
27. Akashi M, Loussararian AH, Adelman DC, Saito M, Koeffler HP: Role

of lymphotoxin in expression of interleukin 6 in human fibroblasts. J Clin Invest 85:121–129, 1990

28. Kahari VM, Heino J, Vuorio E: Interleukin-1 increases collagen production and mRNA levels in cultured skin fibroblasts. Biochem Biophys Acta 929:142–147, 1987

29. Postlethwaite AE, Raghow R, Stricklin GP, Poppleton H, Seyer JM, Kang AH: Modulation of fibroblast functions by interleukin 1: Increased steady-state accumulation of type 1 procollagen mRNAs and stimulation of other functions but not chemotaxis by human recombinant interleukin 1-alpha and beta. J Cell Biol 106:311–318, 1988

30. Goldring MB, Krane SM: Modulation by recombinant interleukin 1 of synthesis of types I and II collagens and associated procollagen mRNA levels in cultured human cells. J Biol Chem 262:16724–16729, 1987

31. Heino J: Connective tissue components in synovial fibroblast cultures exposed to interleukin 1 and prostaglandin E_2. Virchows Arch (Cell Pathol) 50:313–320, 1986

32. Seyer JM, Kang AH: Structural proteins: collagen, elastin and fibronectin. In: Kelley WN, Harris ED, Ruddy S, Sledge CB (eds.). Textbook of Rheumatology, 2nd ed. Philadelphia, WB Saunders, 1985, pp 211–237

33. Ramirez F, De Wet W: Molecular biology of human fibrillar collagen genes. Ann NY Acad Sci 543:109–116, 1988

34. Gearing AJH, Leung H, Bird CR, Thorpe R: Presence of the inflammatory cytokines IL1, TNF, and IL6 in preparations of monoclonal antibodies. Hybridoma 8:361–367, 1989

35. Cicco NA, Lindemann A, Content J, Vandenbussche P, Lubbert M, Gauss J, Mertelsmann R, Herrmann F: Inducible production of interleukin 6 by human polymorphonuclear neutrophils: role of granulocyte-macrophage colony stimulating factor and tumor necrosis factor. Blood 75: 2049–2052, 1990

36. Hilbert DA, Canco MP, Scherle PA, Nordan RP, Van Snick J, Gerphard W, Rudikoff S: T cell derived IL6 is differentially required for antigen specific antibody secretions by primary and secondary B cells. J Immunol 143:4019–4024, 1989

37. Kupper TS, Min K, Sehgal P, Mizutani H, Birchall N, Ray A, May L: Production of IL6 by keratinocytes. Ann NY Acad Sci 557:454–464, 1989

38. Duncan MR, Berman B: Effect on human recombinant interleukin 6 on the biosynthetic functions of cultured adult dermal fibroblasts (abstr). J Invest Dermatol 92:422, 1989

39. Kupper TS, Kronberger E, Bauer EA: Induction of collagenase gene expression in dermal fibroblasts by recombinant and purified fibroblast derived interleukin 6 (IL-6): a potential autocrine pathway for fibroblast collagenase production (abstr). Clin Res 37:529A, 1989

40. Bhardwaj N, Santhanam N, Lau LL, Tatter SB, Ghrayeb J, Rivelis M, Steinman RM, Sehgal PB, May LT: IL6/IFN-B_2 in synovial effusions of patients with rheumatoid arthritis and other arthritides. J Immunol 143:2153–2159, 1989

41. Waage A, Halstensen A, Shalaby R, Brandtzaeg P, Kierulf P, Espevik T: Local production of tumor necrosis factor α, interleukin 1, and interleukin 6 in meningococcal meningitis. J Exp Med 170:1859–1867, 1989

42. Fleischmajer R, Perlish JS, Duncan MR: Scleroderma: a model for fibrosis. Arch Dermatol 119:957–962, 1983

43. Murrary JC, Pollack SV, Pinnell SR: Keloids: a review. J Am Acad Dermatol 4:464–470, 1981

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