Differential Regulation of Collagen, Glycosaminoglycan, Fibronectin, and Collagenase Activity Production in Cultured Human Adult Dermal Fibroblasts by Interleukin 1-Alpha and Beta and Tumor Necrosis Factor-Alpha and Beta

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In order to clarify the role played by immunologically derived cytokines in dermal connective tissue synthesis and degradation, we investigated the effect of human recombinant (hu-r) interleukin (IL) 1-alpha and beta, hu-r tumor necrosis factor (TNF)-alpha and beta, hu-r IL 2, and hu-r granulocyte-macrophage colony-stimulating factor (GM-CSF) on the production of collagen, glycosaminoglycan, fibronectin, and collagenase activity by three lines of cultured human adult dermal fibroblasts. Our results show that 24–72 h treatment of confluent fibroblast cultures with IL 1-alpha or beta or TNF-alpha or beta causes concentration (1 to 1×10^4 U/ml) dependent increases in collagen, glycosaminoglycan, and collagenase activity production, but decreases in fibronectin production. In contrast, treatment with IL 2 and GM-CSF had no effect on fibroblast functions. The data show that IL 1-alpha and beta and TNF-alpha and beta differentially regulate fibroblast functions, and that increases in catabolic functions like collagenase activity production are more than tenfold greater than increases in anabolic functions like collagen production. When these results are considered along with other reports, they suggest that IL 1 and TNF may play predominately a catabolic role *in situ* during dermal fibrotic responses by directly inhibiting fibronectin production and indirectly causing the degradation of collagen and glycosaminoglycan by significantly increasing dermal fibroblast elaboration of collagenase and proteoglycanase activities. *J Invest Dermatol 92:699–706, 1989*

Fibroblasts are responsible for the production and maintenance of the connective tissue matrix. Under basal conditions, the growth and synthetic activities of resident fibroblasts are of a limited nature and are likely controlled only by interaction with native connective tissue matrix components [1,2]. However, following either traumatic or pathologic tissue injury, resident fibroblasts appear to undergo a metabolic activation as cultured fibroblasts derived from sites of ongoing connective tissue metabolism exhibit elevated activities. Fibroblasts derived from sites of anabolic pathologic fibrosis, such as keloid or scleroderma skin, display activated phenotypes characterized by increased production of the connective tissue matrix components, collagens [3–7], glycosaminoglycans [4,8,9], and fibronectin [7], while fibroblasts derived from sites of catabolic pathologic fibrosis, such as rheumatoid synovium or cartilage, produce increased amounts of connective tissue degrading enzymes like collagenase and other neutral proteases, as well as elevated quantities of prostaglandin E_2 [10]. Unfortunately, fibroblasts derived from traumatic wound tissue have seldom been studied, although granulation tissue fibroblasts reportedly differ from normal in the amount and character of glycosaminoglycans secreted [11].

Studies investigating the mechanisms responsible for fibroblast activation have recently focused on the role played by fibroblast-stimulatory cytokines as the early inflammatory phase of fibrotic responses is characterized by the infiltration of injured connective tissue by platelets, neutrophils, monocytes/macrophages, and/or T lymphocytes [12–14]. While evidence of the presence of fibroblast-stimulatory cytokines *in situ* is limited [15–17], numerous *in vitro* studies demonstrate that cytokines can stimulate the growth, directional migration, and/or connective tissue matrix component synthesis/catabolism of cultured fibroblasts. To date, cytokines reported to stimulate fibroblast functions *in vitro* include: transforming growth factor (TGF)-beta, which is produced by platelets, monocytes/macrophages and T lymphocytes [18–21]; platelet-derived growth factor from platelets and monocytes/macrophages [20,22–24]; interleukin (IL)-1, tumor necrosis factor (TNF)-alpha and other less defined monokines from monocytes/macrophages [25–

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Abbreviations:
DME: Dulbecco's modified Eagle's medium
dpm: disintegrations per minute
FCS: fetal calf serum (heat inactivated)
GM-CSF: granulocyte-macrophage colony stimulating factor
hu-r: human-recombinant
IFN: interferon
IL: interleukin
SD: standard deviation
TGF: transforming growth factor
TNF: tumor necrosis factor

30]; TGF-alpha, epidermal growth factor, and other less defined cytokines from platelets [20,31,32]; TNF-beta several less defined fibroblast-stimulatory cytokines from T lymphocytes [33–37] or neutrophils [38]; and other well defined cytokines such as acidic and basic fibroblast growth factor and somatomedin C, whose cellular sources are uncertain [23,39].

Of all the fibroblast-stimulatory cytokines reported, monocyte/macrophage-derived IL 1 has been the most extensively studied, especially in regard to its effect on synovial fibroblasts and chondrocytes. IL 1 has been reported to increase all studied synovial fibroblast and chondrocyte functions, including growth [40], collagen production [26,41] glycosaminoglycan production [42], fibronectin production [26], collagenase production [27,43], proteoglycanase production [43], and prostaglandin E_2 production [27,29]. The ability of IL 1 to elevate multiple functions of synovial fibroblasts and chondrocytes strongly suggests that IL 1 plays a role in the degradation of joint connective tissue seen in arthritic diseases. However, the role of IL 1 in dermal fibrotic diseases, such as keloids or scleroderma, or in normal dermal wound repair is less certain, as the effect of IL 1 on dermal fibroblast function has not yet been comprehensively studied. Although it has long been known that IL 1 increases adult and infant foreskin dermal fibroblast growth [25], it has only recently been reported that IL 1 affects other functions of infant foreskin [29,44] and adult dermal fibroblasts [27].

In this report, we have studied the effect of highly purified samples of human recombinant DNA-derived (hu-r) IL 1-alpha and IL 1-beta on three previously uninvestigated human adult dermal fibroblast functions (collagen, glycosaminoglycan, and fibronectin production) and one previously investigated function (collagenase activity production), in order to clarify the role IL 1 plays in adult dermal connective tissue synthesis and degradation. We similarly investigated the effect of hu-r TNF-alpha and beta as TNF-alpha reportedly increases the growth, collagenase activity production, and prostaglandin E_2 production of infant foreskin dermal fibroblasts [28,29], while TNF-beta has been reported to increase the growth of infant foreskin fibroblasts and the glycosaminoglycan production of lung fibroblasts [33,34]. The effects of two other cytokines, hu-r IL 2 and hu-r granulocyte-macrophage colony-stimulating factor (GM-CSF) were also studied in order to determine if other mediators involved in the immunologic cytokine network also play a role in regulating dermal connective metabolism. Our results demonstrate that IL 1 alpha and beta, and TNF-alpha and beta differentially regulate the four studied fibroblast functions, increasing collagen, glycosaminoglycan, and collagenase activity production but decreasing fibronectin production, while IL 2 and GM-CSF had no effect on any dermal fibroblast function studied.

MATERIALS AND METHODS

Cytokines Hu-r interferon (IFN)-alpha$_2$ (SCH30500; specific activity, 1.2×10^8 U/mg protein) from *Escherichia coli* was kindly supplied by Schering Corporation (Kenilworth, NJ). Hu-r-*E. coli*-derived TNF-alpha (5.0×10^7 U/mg) and TNF-beta (2.0×10^8 U/mg) were gifts from Genentech Inc. (South San Francisco, CA). *E. coli*-derived hu-r IL 1 alpha and beta (1×10^8 U/mg), yeast-derived hu-r IL 2 (2×10^8 U/mg), and yeast-derived hu-r GM-CSF (5×10^7 U/mg) were obtained from Genzyme Corporation (Boston, MA). The titer of these cytokine preparations ranged from 1×10^3 to 6.6×10^7 U/ml of phosphate buffered saline and they were added directly to fibroblast cultures after dilution to the desired concentrations with Dulbecco's Modified Eagle's medium (DME) containing 0.1% human serum albumin. TGF-beta from human platelets (>95% pure) was obtained as a 2.2×10^6 pM solution in 20% acetonitrile: 0.1% trifluoroacetic acid from Calbiochem Biochemicals (San Diego, CA), and after an initial 1:10 dilution in 4 mM HCl it was diluted in DME plus albumin and added directly to cultures.

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 female, line 2 from the facial skin of a 52-year-old male, and line 3 from the facial skin of a 42-year-old female. The fibroblasts were maintained in DME 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-MA Bioproducts, Walkersville, MA) at 37°C in a 5% CO_2 humidified atmosphere. Fibroblast cultures were subcultured by trypsinization and used between the second and tenth passages. We have previously reported on the growth and collagen production of these fibroblast lines and that they respond equally well to IFN treatment in early (second) or later (ninth) passage [45]. We have similarly found the response of these fibroblast lines to treatment with IL 1 and TNF to be independent of passage number between the second and tenth passages.

Assay of Fibroblast Collagen Production Assay of collagen production during a 24-h period by steady-state, confluent fibroblasts cultured in the presence of ascorbic acid and no FCS or 10% FCS was assessed by ^{3}H-proline incorporation into collagenous protein. The use of confluent cultures minimizes growth-related events. Freshly trypsinized fibroblasts were plated in triplicate in 96 well microcultures (0.45 cm^2 surface area; Microtest III; Falcon Labware, Oxnard, CA) at a near confluent density of 25–40,000 fibroblasts per well in 200 μl DME-10% FCS and incubated for 48–120 h to produce a totally confluent monolayer of fibroblasts. The medium was then removed and replaced with 200 μl DME alone or DME plus 10% FCS containing 50 μg/ml ascorbic acid with or without cytokines, and incubated for an additional 24, 48, or 72 h. Cultures were pulsed with 0.5 μCi of ^{3}H-proline (31 Ci/mmol; Amersham Corp, Arlington Heights, IL), 50 μg/ml beta-aminopropionitrile, and 50 μg/ml 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 [46–48].

Briefly, extracellular ^{3}H-proline-labeled collagen was extracted by the addition of acetic acid-pepsin to the culture media in each microwell and purified by successive salt precipitations at acid and neutral pH. The final washed precipitate was solubilized in 0.5 M acetic acid, incorporated into a scintillation cocktail, and counted in a liquid scintillation counter. In our hands this assay yields material that is 95% collagenase digestible, and the method isolates less than 1% of non-collagenous proteins biosynthetically labeled with ^{3}H-tryptophan [48]. Each experimental condition was done in triplicate wells and averaged results are expressed as disintegrations per minute (dpm) of ^{3}H-collagen per 10^3 cells ± standard deviation (SD), with cell numbers determined by hemocytometer-counting the number of trypsin released cells present in a series of identically treated microcultures.

Assay of Fibroblast Glycosaminoglycan Production Microcultures treated identically to those described above for assay of collagen production were used to assess glycosaminoglycan production except they were incubated without beta-aminopropionitrile and labeled with 0.5 μCi of ^{3}H-glucosamine (22 Ci/mmol, Amersham) for the terminal 24 h of culture. The ^{3}H-glucosamine incorporation into cetylpyridinium chloride-precipitated glycosaminoglycans in both the culture media and cell layer was then determined as previously described [8,49]. Briefly, cellular and supernatant protein was digested by incubation at 55°C for 4 h after the addition of pronase (Calbiochem) to the culture media of each microwell. After centrifugation to clear and the addition of carrier chondroitin sulfate and hyaluronic acid (Sigma Chemical, St. Louis, MO), glycosaminoglycans were precipitated by the addition of cetylpyridinium chloride to a final concentration of 0.5%. Precipitated material was collected on glass fiber filters, washed, dried, and the ^{3}H-glucosamine-labeled glycosaminoglycans bound to the filters quantitated by liquid scintillation spectrometry. Each experimental condition was done in triplicate wells and averaged results are expressed as dpm of ^{3}H-glycosaminoglycan per 10^3 cells ± SD,

with cell numbers determined by hemocytometer-counting the number of trypsin released cells present in a series of identically treated microcultures.

Assay of Fibroblast Fibronectin Production Near confluent fibroblast cultures were set up by placing 100–160,000 freshly trypsinized fibroblasts, contained in 1 ml of DME-10% FCS, into 24 well microcultures (2.0 cm^2 surface area; Linbro; Flow Labs, McLean, VA) and incubating for 48–120 h at 37°C in a 5% CO_2 atmosphere to permit growth to total confluency. Medium was then removed and replaced with 0.5 ml fresh DME alone or DME + 10% FCS containing 50 μg/ml ascorbic acid with or without cytokines and incubated for an additional 24, 48, or 72 h. Supernatant medium was removed and the fibronectin content of the medium was determined using a commercially available immunoturbidimetric assay kit (Boehringer-Mannheim, Indianapolis, IN). This assay uses an antisera to human fibronectin which 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 triplicate wells, and averaged results obtained by measuring the fibronectin concentration of 0.3 ml aliquots of supernatant medium are reported as μg per 10^5 cells ± SD, with cell numbers being determined by counting the number of trypsin-released cells per well using a hemocytometer.

Assay of Fibroblast Collagenase Activity Production Twenty-four well microcultures treated identically to those described above for assay of fibronectin production were used to assess collagenase activity production. However, terminal 24, 48, or 72 h incubation were performed in only ascorbic acid supplemented DME alone, as FCS interfered with the subsequent assay of culture supernatant collagenase activity. The supernatants from these cultures, after activation by brief trypsin treatment, were then assayed for collagenase activity by measuring the solubilization of ^{3}H-labeled rat (type I) collagen fibrillar gels using previously reported methods [44,50,51] modified to assay larger volumes of supernatant over a longer period of time, in order to accurately quantitate the lower levels of collagenase activity produced by control fibroblast cultures.

Briefly, the procollagenase in 1.0 ml of culture supernatant or dilutions of supernatant in DME was activated by incubation at 28°C for 12 min with 50 units of trypsin (DPCC-treated; Sigma), followed by the addition of a tenfold excess of soybean trypsin inhibitor (Sigma) to inhibit further trypsin activity. ^{3}H-labeled-fibrillar collagen gels were formed by mixing 800 μl of a 1.25 mg/ml solution of unlabeled rat tail (type I) collagen (Sigma) in 0.00IN acetic acid with 10 μl of ^{3}H-propionylated-rat tail collagen (0.51 mCi/mg; New England Nuclear, Boston, MA) in 0.0IN acetic acid and neutralizing with 200 μl of fivefold concentrated DME. Twenty-five microliter (25 μg collagen; 30,000 dpm) aliquots of this neutralized solution were then dispensed in 24-well plates (Linbro) tilted at a 45° angle and allowed to gel at 37°C in a 5% CO_2 humidified atmosphere for 4 h. Formed fibrillar gels adherent to the edges of the well bottom were then washed twice with DME and incubated for an additional 18 h in 1 ml of DME. After removal of this 18-h DME wash, the collagenolytic activity of trypsin-activated supernatant samples was then determined by placing 1.0 ml of sample in each well and incubating for a further 20 h. Each milliliter of fluid was then carefully removed from the wells and subjected to scintillation counting to determine the amount of collagen solubilized from each gel. The collagenase activity of each sample was then calculated from the percentage gel lysis by reference to 100% digestion caused by 100 units/ml of a highly purified clostridial collagenase (Sigma) with 1.0 ml of control fibroblast culture supernatant typically causing approximately 20%–30% gel lysis. Supernatants were assayed in triplicate and averaged results are reported as mU of collagenase activity per 10^5 cells ± SD, with 1.0 U defined as the amount of collagenase activity digesting 1.0 μg of collagen per minute. To ensure that gels were not composed of denatured collagen, which is digested by other proteolytic enzymes besides collagenase, each batch of gels was tested for sensitivity to digestion by 50 U/ml of trypsin, which typically caused less than 10% gel lysis over a 20-h period.

RESULTS

Effect of Cytokines on Fibroblast Collagen Production Samples of hu-r cytokines were assayed for effect on the collagen production of adult dermal fibroblasts using culture conditions (DME plus 10% FCS) identical to those we had previously employed to demonstrate IFN-mediated inhibition of collagen production [45]. The use of confluent cultures minimizes growth related events, while the use of 10% FCS ensures optimal levels of collagen production. Because the optimal collagen production occuring in the presence of 10% FCS may be partially due to direct stimulation by serum factors such as TGF-beta [18–20], studies were also performed in the absence of FCS to assess the effect of cytokines on basal collagen production.

Cytokines were first tested on fibroblast line 1 using a 48-h treatment period to determine maximum effective concentrations. As detailed in Table I, IL 1-alpha and beta and TNF-alpha and beta caused concentration-dependent increases in collagen production in the absence of FCS but had no effect on collagen production in the presence of FCS. The reason for ineffective stimulation of collagen production in the presence of 10% FCS was not investigated, but may be related to the ability of IL 1 and TNF to induce the growth of confluent fibroblast cultures in the presence but not the absence of FCS [25,28]. While we observed that IL 1 and TNF had no effect on the growth of confluent microcultures in the absence of FCS, IL 1 and TNF frequently induced 20%–30% increases in cell numbers

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

		^{3}H-Collagen[b] (DPM/10^3 Cells ± SD)	
Cytokine	Concentration	DME	DME + 10% FCS
None		45 ± 2	119 ± 12
r IL 1-α	1 U/ml	55 ± 5	115 ± 2
	10	70 ± 7	110 ± 4
	10^2	79 ± 2	123 ± 13
r IL 1-β	1 U/ml	50 ± 5	116 ± 12
	10	76 ± 5	119 ± 8
	10^2	91 ± 10	110 ± 14
r TNF-α	1 U/ml	49 ± 4	125 ± 12
	10	55 ± 6	114 ± 4
	10^2	75 ± 8	103 ± 15
	10^4	84 ± 4	95 ± 10
r TNF-β	1 U/ml	44 ± 3	115 ± 20
	10	51 ± 9	127 ± 16
	10^2	62 ± 5	105 ± 8
	10^4	74 ± 7	113 ± 9
r IL 2	10 U/ml	47 ± 4	112 ± 8
	10^2	43 ± 1	105 ± 10
	10^3	46 ± 5	112 ± 14
r GM-CSF	10 U/ml	47 ± 3	118 ± 8
	10^2	48 ± 1	106 ± 12
	10^3	43 ± 2	113 ± 15
r IFN-α_2	10^4 U/ml	21 ± 3	66 ± 6
TGF-β	10^3 pM	246 ± 20	378 ± 35

[a] Representative experiment showing the effect of 48 h of cytokine exposure on the collagen production of adult dermal fibroblast line 1 cultured in the absence and presence of FCS as described in *Materials and Methods*. Data were confirmed in two additional experiments.

[b] Collagen production was assessed by measuring ^{3}H-proline incorporation into pepsin-resistant, salt-precipitated extracellular collagen and is expressed as dpm/10^3 cells ± SD of triplicate determinations. All IFN-alpha$_2$ and TGF-beta treatment values are significantly different from untreated control values (Student's two-tailed *t*-test: $P < 0.05$) as are treatment values in DME alone for IL 1 alpha and beta at 10 U/ml or greater and TNF-alpha and beta at 10^2 U/ml or greater. All other treatment values are not significantly different from untreated control values.

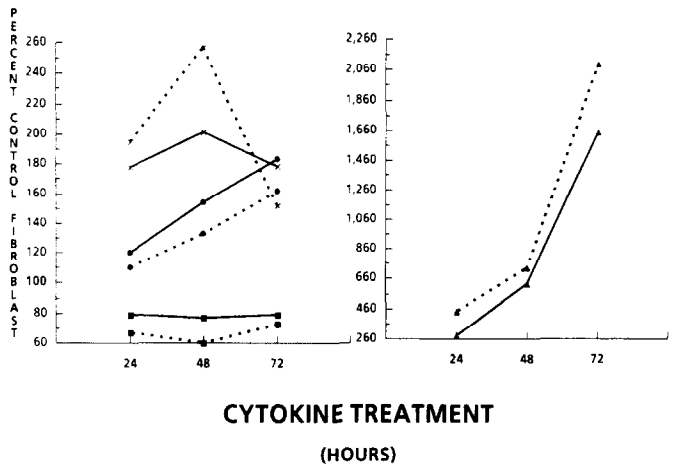

Figure 1. Effect of exposure time on cytokine modulation of adult dermal fibroblast functions. Confluent cultures of fibroblast line 1 incubated in DME without FCS were exposed to 10 U/ml of IL 1-beta *(solid line)* or 10^2 U/ml of TNF-alpha *(broken line)* for 24, 48, or 72 h. Collagen *(closed circle)*, glycosaminoglycan *(X)*, fibronectin *(closed square)* and collagenase activity *(closed triangle)* production were assessed as described in *Materials and Method* and are expressed as percentage of control fibroblast production (100%). Control fibroblast values ± SD at 24, 48, and 72 h were collagen: 49 ± 3.3, 31.9 ± 1.7, 24.2 ± 0.4 dpm/10^3 cells; glycosaminoglycan: 43.7 ± 2.6, 39.2 ± 1.1, 24.9 ± 0.2 dpm/10^3 cells; fibronectin: 0.82 ± .04, 1.44 ± .06, 1.98 ± .13 μg/10^5 cells; collagenase activity: 5.24 ± .56, 6.88 ± .44, 2.65 ± .21 mU/10^5 cells. All plotted cytokine treatment values are significantly different from untreated control values. (Student's two tailed *t*-test: $P < 0.05$) except TNF-alpha at 24 hours.

over a 48-h period in the presence of 10% FCS. The decreased basal collagen production (on a per cell basis) of such post-confluent cultures [52] may compensate for any increase induced by IL 1 or TNF resulting in no net increase in collagen production when compared to confluent untreated controls. However, if highly confluent microcultures are not used, IL 1 and TNF induce 60%–70% increases in cell numbers resulting in a net decrease in collagen production on a per cell basis (data not shown). TNF-alpha has recently been reported to decrease the collagen production of infant foreskin fibroblasts cultured in 10% FCS [53].

At the highest concentrations tested, IL 1 and TNF caused 64%–102% increases in collagen production in the absence of FCS. The IL 1s, however, were more potent stimulators than TNFs, as 10 U/ml (5.7 pM) of the IL 1s caused 56%–68% increases, while 100 U/ml (117 pM) of TNF-alpha or 1000 U/ml (270 pM) TNF-beta were required to induce a similar increase in collagen production. In contrast to the stimulatory activity of IL 1 and TNF, IL 2 and GM-CSF had no effect on collagen production when tested at concentration up to 10^3 U/ml either in the absence or presence of FCS. A known inhibitor, IFN-alpha$_2$, and a known stimulator, TGF-beta, of collagen production were also assayed to confirm the response of our culture and assay systems [18–20,45,48]. As shown in Table I, IFN-alpha$_2$ inhibited and TGF-β stimulated, collagen production in both the absence and presence of FCS, suggesting that our system was capable of detecting increases or decreases in collagen production under both culture conditions. We have not attempted to determine if type I or type III collagen is more affected by IL 1 and TNF treatment. However, considering that known modulators of collagen production coordinately effect both type I and type III collagen production by dermal fibroblasts [35], our results may reflect similar changes.

To determine the kinetics of the response of fibroblast line 1 to IL 1 and TNF treatment, we investigated collagen production after 24, 48, and 72 h cytokine exposure. As shown in Fig 1, in the absence of FCS, IL 1-beta and TNF-alpha caused progressively greater increases in collagen production with time. IL 1-beta and TNF-alpha in the presence of 10% FCS had no effect on collagen production (data not shown).

To ensure that the effects of IL 1 and TNF were not restricted to one fibroblast line, the influence of these cytokines was examined on two additional adult dermal fibroblast lines. As shown in the detail in Table II, fibroblast lines 2 and 3 responded similarly to fibroblast line 1, with IL 1-alpha and beta and TNF-alpha and beta stimulating collagen production in the absence but not in the presence of FCS. Because the three fibroblast lines tested were derived from skin taken from different anatomical sites (breast, face) of both

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

		Fibroblast line 2		Fibroblast line 3	
Assay	Cytokine	DME	DME + 10% FCS	DME	DME + 10% FCS
Collagen (DPM/10^3 cells ± SD)	None	26 ± 2	48 ± 4	32 ± 1	78 ± 7
	r IL 1-α	35 ± 1	46 ± 4	118 ± 13	79 ± 8
	r IL 1-β	36 ± 2	53 ± 8	100 ± 11	75 ± 2
	r TNF-α	36 ± 3	44 ± 4	45 ± 2	77 ± 5
	r TNF-β	32 ± 1	44 ± 1	45 ± 8	74 ± 4
Glycosaminoglycan (DPM/10^3 cells ± SD)	None	61 ± 4	100 ± 13	46 ± 7	66 ± 2
	r IL 1-α	172 ± 18	206 ± 12	70 ± 5	156 ± 22
	r IL 1-β	109 ± 6	234 ± 28	59 ± 2	107 ± 12
	r TNF-α	152 ± 6	222 ± 8	99 ± 3	123 ± 1
	r TNF-β	150 ± 4	216 ± 4	71 ± 2	80 ± 1
Fibronectin (μg/10^5 cells ± SD)	None	1.91 ± 0.16	2.34 ± 0.18	1.77 ± 0.06	1.92 ± 0.10
	r IL 1-α	1.19 ± 0.07	1.68 ± 0.12	1.44 ± 0.04	1.55 ± 0.03
	r IL 1-β	1.36 ± 0.03	1.89 ± 0.07	1.60 ± 0.08	1.69 ± 0.01
	r TNF-α	1.12 ± 0.16	1.41 ± 0.09	1.25 ± 0.02	1.42 ± 0.06
	r TNF-β	1.16 ± 0.09	1.68 ± 0.05	1.33 ± 0.02	1.22 ± 0.03
Collagenase (mU/10^5 cells ± SD)	None	11.5 ± 1.4	ND[b]	2.2 ± 0.4	ND
	r IL 1-α	85.7 ± 5.9	ND	6.5 ± 0.2	ND
	r IL 1-β	132.6 ± 2.0	ND	34.7 ± 0.7	ND
	r TNF-α	74.3 ± 4.1	ND	8.3 ± 1.3	ND
	r TNF-β	80.6 ± 5.0	ND	4.9 ± 0.7	ND

[a] Experiments show the effect on 48 h of cytokine exposure on the biosynthetic functions of adult dermal fibroblast lines 2 and 3 cultured in the absence and presence of FCS. Confluent fibroblast cultures were exposed to 10 U/ml of IL 1-alpha or beta or 10^2 U/ml of TNF-alpha or beta and collagen, glycosaminoglycan, fibronectin, and collagenase activity production assessed as described in *Materials and Methods*. All listed cytokine treatment values are significantly different from untreated control values (Student's two tailed *t*-test: $P < 0.05$) except values for collagen production in the presence of FCS.

[b] ND: not done.

males and females, the data suggest that IL 1 and TNF will similarly affect the collagen production of most adult dermal fibroblast lines.

Effect of Cytokines on Fibroblast Glycosaminoglycan Production The effect of hu-r cytokines on adult dermal fibroblast glycosaminoglycan production was studied using culture conditions identical to the collagen assay. Assays were again performed in the presence and absence of FCS, as serum factors such as TGF-beta also stimulate glycosaminoglycan production [19]. As shown in Table III, 48-h treatments with IL 1-alpha and beta and TNF-alpha and beta caused concentration-dependent increases in the glycosaminoglycan production of fibroblast line 1 both in the absence and presence of FCS, while IL 2 and GM-CSF were without effect. At the highest concentrations tested, IL 1 and TNF caused 84%–119% increases in glycosaminoglycan production in the presence of FCS, while in the absence of FCS they caused 102%–202% increases. As found with collagen production IL 1s were more potent stimulators than TNFs, as 10 U/ml (5.7 pM) of IL 1-alpha or beta stimulated as much glycosaminoglycan production as 100 U/ml (117 or 27 pM) of TNF-alpha or beta. Known modulators of glycosaminoglycan production also acted in both the absence and presence of FCS, with IFN-alpha$_2$ suppressing production equally well in both systems, while TGF-beta caused increases in glycosaminoglycan production, which, like IL 1 and TNF, were more significant in the absence (320%) than presence (115%) of FCS [19,54]. We have not attempted to determine which individual types of glycosaminoglycans are affected by the cytokine treatments. However, considering that only 50% to 60% of the ^{3}H-glucosamine-labeled glycosaminoglycans produced by adult dermal fibroblasts is hyaluronic acid [8,36] our results may reflect changes in the production of either hyaluronic acid, sulfated glycosaminoglycans, or heparin, although

Table III. Effect of Cytokines on Glycosaminoglycan Production by Dermal Fibroblast Line 1[a]

Cytokine	Concentration	^{3}H-Glycosaminoglycan[b] (DPM/10^3 Cells ± SD) DME	DME + 10% FCS
None		49 ± 2	121 ± 4
r IL 1-α	1 U/ml	53 ± 4	130 ± 6
	10	72 ± 5	190 ± 13
	10^2	132 ± 2	261 ± 18
r IL 1-β	1 U/ml	48 ± 6	127 ± 5
	10	78 ± 7	207 ± 17
	10^2	148 ± 8	232 ± 18
r TNF-α	1 U/ml	46 ± 5	131 ± 10
	10	57 ± 4	145 ± 7
	10^2	75 ± 9	202 ± 7
	10^4	121 ± 4	266 ± 25
r TNF-β	1 U/ml	47 ± 2	107 ± 2
	10	64 ± 7	120 ± 11
	10^2	82 ± 9	164 ± 12
	10^4	99 ± 2	223 ± 17
r IL 2	10 U/ml	51 ± 5	114 ± 11
	10^2	47 ± 3	125 ± 5
	10^3	48 ± 4	132 ± 10
r GM-CSF	10 U/ml	50 ± 4	116 ± 4
	10^2	52 ± 6	127 ± 4
	10^3	49 ± 2	144 ± 5
r IFN-α_2	10^4 U/ml	28 ± 3	56 ± 3
TGF-β	10^3 pM	206 ± 25	260 ± 12

[a] Representative experiment showing the effect of 48 h of cytokine exposure on the glycosaminoglycan production of adult dermal fibroblast line 1 cultured in the absence and presence of FCS as described in *Materials and Methods*. Data were confirmed in three additional experiments.

[b] Glycosaminoglycan production was assessed by measuring ^{3}H-glucosamine incorporation into cetylpyridinium chloride precipitated material in both culture media and cell layer and is expressed as dpm/10^3 cells ± SD of triplicate determinations. All listed treatment values are significantly different from untreated control values (Student's two-tailed *t*-test: $P < 0.05$) except IL 1-alpha, beta and TNF-alpha and beta at 1 U/ml, TNF-beta 10 U/ml in 10% FCS and all concentrations of IL 2 and GM-CSF.

TNF-alpha and beta stimulate only the hyaluronic acid production of adult lung fibroblasts [34].

As shown in Fig 1, IL 1-beta and TNF-alpha also increased the glycosaminoglycan production of fibroblast line 1 after 24 and 72 h of cytokine exposure in the absence of FCS, with 72 h augmentation being less than 48 h values. Similar effects were observed in the presence of 10% FCS (data not shown). IL 1 and TNF also stimulated the glycosaminoglycan production of fibroblast lines 2 and 3 as detailed in Table II.

Effect of Cytokines on Fibroblast Fibronectin Production The effect of hu-r cytokines on adult dermal fibroblast fibronectin production was studied using culture conditions identical to the collagen and glycosaminoglycan assays but employing larger cultures (24 well plates), as greater than 200 μl of supernatant per sample was required for subsequent immunoturbidimetric assay of fibronectin. Assays were again performed in the presence and absence of FCS as serum factors such as TGF-beta also stimulate fibronectin production [18]. As shown in Table IV, IL 1-alpha and beta and TNF-alpha and beta caused concentration-dependent decreases in the fibronectin production of fibroblast line 1 both in the absence and presence of FCS, with IL 1 and TNF being approximately equipotent, as 100 U/ml (IL 1s: 57 pM; TNF-alpha: 117 pM; TNF-beta: 27 pM) of each cytokine caused 35% to 55% inhibition of fibronectin production. As with collagen and glycosaminoglycan production, IL 2 and GM-CSF were similarly ineffective in modulating fibroblast fibronectin production. The two known modulators of dermal fibroblast functions, IFN-alpha$_2$ and TGF-beta, behaved as previously reported with IFN-alpha$_2$ having no effect and TGF-beta increasing fibronectin production in both the absence and presence of FCS [18,54].

When the fibronectin production of fibroblast line 1 cultured in the absence of FCS was examined after 24, 48, or 72 h of IL 1-beta or TNF-alpha exposure, fibronectin levels were found to be decreased by approximately the same amount at each time point (Fig 1). Similar effects were observed in the presence of 10% FCS (data not shown). As shown in Table II, IL 1 and TNF also depressed the fibronectin production of fibroblast lines 2 and 3. Thus in contrast to stimulating collagen and glycosaminoglycan production, IL 1-alpha and beta, and TNF-alpha and beta inhibit fibronectin production.

Earlier studies done with synovial or infant foreskin fibroblasts both agree and conflict with our observation that IL 1 decreases adult dermal fibroblast fibronectin production. Earlier studies performed with partially purified mononuclear cell-derived IL 1 preparations showed increases in extracellular fibronectin production by synovial fibroblasts [26]. However, such crude IL 1 preparations may have contained other mononuclear cell products, such as TGF-beta, which could have masked the inhibitory effect of IL 1 by stimulating fibronectin production [18]. This seems likely as hu-r-IL 1-alpha and beta have recently been reported to decrease the extracellular fibronectin produced by infant foreskin fibroblasts without affecting fibronectin mRNA levels [55], and ultrapure preparations of mononuclear cell-derived IL 1 have been shown to decrease synovial fibroblast fibronectin production [56].

Effect of Cytokines on Fibroblast Collagenase Activity Production We also investigated the influence of cytokines on the net collagen degrading enzymatic activity released by cultured adult dermal fibroblast in order to determine if cytokines play a role in dermal connective tissue degradation. Our assay of trypsin-activatable collagenolytic activity measures net collagenase activity, as fibroblast supernatant media contains both the enzyme collagenase and its inhibitor, tissue inhibitor of metalloproteinases, and both have been shown to be induced by IL 1 [55]. The results illustrated in Table V show that fibroblast line 1 cultured in the absence of FCS produced significantly elevated quantities of collagenase activity when treated for 48 h with IL 1-alpha and beta and TNF-alpha and beta, but unaltered levels of collagenase activity when treated with IL 2 or GM-CSF. The increases in collagenase activity induced by IL 1 and TNF were concentration dependent with the highest

Table IV. Effect of Cytokines on Fibronectin Production by Dermal Fibroblast Line 1[a]

Cytokine	Concentration	Fibronectin[b] ($\mu g/10^5$ Cells ± SD) DME	DME + 10% FCS
None		1.80 ± .04	2.82 ± .17
r IL 1-α	1 U/ml	1.85 ± .23	2.40 ± .14
	10	1.12 ± .16	1.69 ± .09
	10^2	0.86 ± .05	1.44 ± .08
r IL 1-β	1 U/ml	1.78 ± .18	2.87 ± .19
	10	1.18 ± .04	2.03 ± .05
	10^2	0.93 ± .10	1.29 ± .07
r TNF-α	1 U/ml	1.65 ± .20	2.62 ± .15
	10	1.20 ± .14	1.82 ± .06
	10^2	1.05 ± .07	1.69 ± .15
	10^4	0.94 ± .09	1.52 ± .11
r TNF-β	1 U/ml	1.75 ± .14	2.99 ± .25
	10	1.45 ± .13	2.03 ± .19
	10^2	1.17 ± .10	1.57 ± .15
	10^4	1.06 ± .05	1.21 ± .13
r IL 2	10 U/ml	1.73 ± .19	2.65 ± .19
	10^2	1.84 ± .17	3.21 ± .14
	10^3	1.92 ± .14	2.93 ± .26
r GM-CSF	10 U/ml	1.59 ± .08	2.76 ± .23
	10^2	1.75 ± .13	3.10 ± .35
	10^3	1.72 ± .10	2.92 ± .17
r IFN-α_2	10^4 U/ml	1.78 ± .09	3.01 ± .20
TGF-β	10^3 pM	3.06 ± .26	4.65 ± .38

[a] Representative experiment showing the effect of 48 h of cytokine exposure on the fibronectin production of adult dermal fibroblast line 1 cultured in the absence and presence of FCS as described in Materials and Methods. Data was confirmed in two additional experiments.

[b] Fibronectin production was assessed by measuring the quantity of fibronectin present in fibroblast culture supernatants using an immunoturbidimetric assay and is expressed as ug/10^5 cells ± SD of triplicate determinations. The TGF-beta treatment value and treatment values for IL 1 alpha and beta and TNF-alpha and beta at 10 U/ml or greater are significantly different from untreated control values (Student's two-tailed *t*-test: $P < 0.05$). All other treatment values are not significantly different from untreated control values.

Table V. Effect of Cytokines on Collagenase Activity Produced by Dermal Fibroblast Line 1[a]

Cytokine	Concentration	Collagenase[b] (mU/10^5 Cells ± SD)
None		3.18 ± .32
r IL 1-α	1 U/ml	32.4 ± 2.1
	10	56.2 ± 4.7
	10^2	62.4 ± 5.6
r IL 1-β	1 U/ml	21.0 ± 3.1
	10	40.8 ± 4.6
	10^2	59.9 ± 4.3
r TNF-α	1 U/ml	12.5 ± 1.5
	10	18.1 ± 1.3
	10^2	31.6 ± 4.1
	10^4	57.1 ± 6.3
r TNF-β	1 U/ml	7.91 ± .30
	10	12.2 ± 1.3
	10^2	19.2 ± .32
	10^4	39.3 ± 5.6
r IL 2	10 U/ml	3.10 ± .29
	10^2	3.08 ± .36
	10^3	3.34 ± .35
r GM-CSF	10 U/ml	3.20 ± .25
	10^2	3.28 ± .34
	10^3	2.80 ± .41
r IFN-α_2	10^4 U/ml	5.60 ± .43

[a] Representative experiment shows the effect of 48 h of cytokine exposure on the collagenase activity production of adult dermal fibroblast line 1 cultured in DME without FCS as described in *Materials and Methods*. Data were confirmed in two additional experiments.

[b] Collagenase activity of trypsin-activated fibroblast culture supernatants was assessed by solubilization of ^{3}H-labeled rat (type I) collagen fibrillar gels and is expressed as mU/10^5 cells ± SD of triplicate determinations. All listed cytokine treatment values are significantly different from untreated control values (Student's two-tailed *t*-test: $P < 0.05$) except all concentrations of IL 2 and GM-CSF.

concentrations tested causing 12.3- to 18.8-fold increases in production. The IL 1 appear to be more potent inducers than TNF as 10–100 U/ml (5.7–57 pM) of IL 1 alpha or beta stimulated as much collagenase production as 10^4 U/ml (11,700 or 2,700 pM) of TNF-alpha or beta. Moreover, IL 1 and TNF were much more effective stimulators of collagenase activity production than of collagen or glycosaminoglycan production, as even 1 U/ml induced 2.5- to 9.1-fold increases in collagenase activity. These increases were also much greater than those caused by a higher concentration of IFN-alpha$_2$, another reported stimulator of collagenase activity production [54].

The significant increases in net collagenase activity released by fibroblast line 1 were time dependent, as IL 1-beta and TNF-alpha treatment caused progressively greater enhancement of collagenase activity production after 24, 48, and 72 h of cytokine treatment (Fig 1). Fibroblast lines 2 and 3 also responded to IL 1 and TNF treatment by producing elevated levels of collagenase activity as shown in Table II.

DISCUSSION

The purpose of this study was to clarify the role played by immunologically derived cytokines in initiating dermal fibrosis by investigating the effect of cytokines on some dermal fibroblast functions that are known to be activated during fibrotic responses; namely, collagen, glycosaminoglycan, fibronectin, and collagenase activity production. Our results suggest that certain immunologically derived cytokines, IL 1-alpha and beta and TNF-alpha and beta, may play a major role in regulating dermal fibrosis, while other cytokines, IL 2 and GM-CSF, likely play little or no role in dermal fibrosis, as they altered none of the fibroblast functions studied. This latter concept is further supported by a previous report demonstrating that IL 2 also has no effect of dermal fibroblast proliferation [25]. Similarly, several previous reports support our observation that IL 1-alpha and beta and TNF-alpha and beta increase collagen, glycosaminoglycan, and collagenase activity production [26,27,34,41–44,55,57] but decrease fibronectin production [55,56]. However, the majority of these previous investigations were performed on infant foreskin, lung, or synovial fibroblasts and chondrocytes rather than on adult dermal fibroblasts, and often employed mononuclear cell-derived IL 1 and TNF preparations of low specific activity, rather than the high specific activity samples of r-IL 1-alpha and beta or r-TNF-alpha and beta used in this report. Our results, therefore, appear to be the first reporting that high specific activity samples of hu-r-IL 1-alpha and beta and TNF-alpha and beta increase the production of collagen, glycosaminoglycan, and collagenase activity but decrease the production of fibronectin by cultured human adult dermal fibroblast, although hu-r IL 1 has recently been shown to increase human infant foreskin fibroblast production of collagen and collagenase activity [29,55]. This report also confirms previous investigations performed on dermal fibroblasts using lower specific activity in IL 1, in that mononuclear cell-derived preparations of IL 1 (approximately 25% IL 1-alpha, 75% IL 1-beta) have been reported to increase the production of collagenase activity and collagen by infant foreskin or adult dermal fibroblasts [29,44,57], while similarly naturally derived TNF-alpha preparations have been shown to increase infant foreskin fibroblast collagenase production [29]. While these previously reported observations could possibly have been ascribed to spurious contaminants in naturally derived cytokine preparations, this report strongly suggests that IL 1-alpha and beta and TNF-alpha were the molecules responsible for these previously observed activities.

Our finding that IL 1-alpha and beta and TNF-alpha and beta have an identical spectrum of activities on dermal fibroblasts agrees well with the shared activities of these two mediators in other biologic systems, as has been recently reviewed [58]. Because IL 1 and

TNF lack homology and act through separate receptors [58], the reason for their overlapping activities in any biologic system is currently uncertain. However, recent reports suggest that TNF-alpha may act by inducing IL 1 production, which could then act in an autocrine manner on the targeted cells [59,60]. Whether TNF-beta also induces IL 1 production has not yet been determined but TNF-alpha and beta do bind to the same cell surface receptor (reviewed in Ref 58). Such a mechanism may explain why TNF-alpha and beta were approximately a log-fold less potent that IL 1-alpha and beta in modulating three of the four fibroblast functions we studied. In contrast, IL 1-alpha and IL 1-beta, which have only 26% homology but act through the same receptor (reviewed in Ref 58), were found to be equipotent in modulating all four of the fibroblast functions we studied. These findings are in agreement with a previous assessment of the activities of IL 1-alpha and beta in three other bioassays [61].

Our findings reveal that IL 1 and TNF differentially regulate cultured adult dermal fibroblast functions and suggest that these cytokines could play both an anabolic and a catabolic role in dermal connective tissue metabolism during fibrotic responses. However, because our results indicate that after 48-h cytokine exposure collagen production at its maximum is increased only 64% – 102% while net collagenase activity is increased by 1230% – 1880%, IL 1 and TNF would likely have a net catabolic effect *in situ*, where, unlike in tissue culture, latent procollagenase is spontaneously activated by other mediators such as plasmin. Similarly, the increased glycosaminoglycan production we have observed with IL 1 and TNF treated fibroblasts (maximum increases: 102% – 202%) may be dominated by increases in proteoglycanase activity, which reportedly ranges up to 490% after stimulation of synovial or dermal fibroblasts or chondrocytes by mononuclear-cell-derived preparations of IL 1 [43,62]. The significant stimulatory effect of IL 1-alpha and beta, and TNF-alpha and beta on the production of collagenase and proteoglycanase activities, along with the observed direct inhibition of dermal fibroblast fibronectin production, strongly suggest that in addition to stimulating fibroblast growth, these cytokines act selectively to activate the catabolic functions of dermal fibroblasts, and thus, they likely play predominately a catabolic role *in situ* during dermal fibrotic responses. While fibroblasts with a catabolic phenotype have yet to be isolated from dermal fibrotic tissue, it is speculated that such fibroblasts play a major role in connective tissue matrix remodeling during the later stages of dermal wound repair [12]. IL 1 and TNF, therefore, may play a role in the progression of dermal wound repair from the earlier anabolic granulation-tissue phase to the later catabolic matrix remodeling phase by converting the anabolic phenotype fibroblasts of the granulation tissue phase to the catabolic phenotype fibroblasts thought to be present in the matrix remodeling phase.

Because IL 1 and TNF treated fibroblasts appear to have a catabolic phenotype, it is thus unlikely that these cytokines are the mediators responsible for inducing the anabolic phenotype fibroblasts isolated from sites of pathologic dermal fibrosis, such as keloid and scleroderma skin [3 – 9]. However, refractoriness to IL 1 or TNF associated mechanisms may be involved in the pathogenesis of scleroderma as both the collagen and procollagen mRNA production by scleroderma fibroblasts and the glycosaminoglycan production of rabbit granulation tissue fibroblasts have recently been shown to be unresponsive to treatment with low concentrations of mononuclear cell-derived IL 1 [11,57]. When these reports [11,55] are considered along with the hypothesis that fibroblast stimulation by IL 1 or TNF may be required for progression from the anabolic granulation tissue phase of dermal wound repair to the catabolic matrix-remodeling phase, it suggests that normal granulation tissue fibroblast acquire *in situ* responsiveness to IL 1 or TNF and convert to a catabolic phenotype but scleroderma fibroblasts fail to make a catabolic conversion resulting in the prolonged and excessive *in situ* deposition of dermal connective tissue that is the hallmark of scleroderma. While such a concept is attractive, more work is clearly needed before the effect of cytokine-mediated fibroblast activation on dermal fibrosis is fully elucidated.

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