Polymorphonuclear Leukocyte and Monocyte Chemoattractants Produced by Human Fibroblasts

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ABSTRACT The elaboration of leukocyte chemotactic factors by human fibroblasts was studied. 12 lines of normal fibroblasts obtained by skin biopsy and then cultured in vitro produced chemoattractants (assessed by modified Boyden-chamber techniques) for both peripheral blood polymorphonuclear leukocytes and monocytes (obtained by Hypaque-Ficoll and dextran sedimentation). Chemotactic activity was not present preformed in fibroblasts, and cycloheximide blocked its elaboration. The chemotactic activity of crude-culture supernate was heat stable (56°C for 30 min), trypsin- and pronase-sensitive, and neuraminidase resistant. Characterization of the chemotactic activity by gel filtration (Sephadex G-75) showed two active fractions, one with mol wt >100,000 and the other <10,000. In studies designed to relate these chemotactic factors to collagen, we have confirmed that type I collagen and α_1-chains are chemotactically active for monocytes but not polymorphonuclear leukocytes. However, the chemotactic activity in fibroblast-culture media was distinct from collagen in that it attracted neutrophils, it was not precipitated by 25% ammonium sulfate, and it was resistant to collagenase treatment; ascorbic acid, in concentrations known to stimulate fibroblast collagen synthesis, had no effect on the elaboration of the chemotactic factors. Furthermore, amino acid analysis of Sephadex G-75 fractions with chemotactic activity failed to reveal amino acids such as hydroxyproline characteristic of collagen. In addition to the chemotactic factors secreted by fibroblasts, a heat-resistant factor (30 min at 56°C) which generated the chemotactically active fragment of C5 (C5a) from human serum was also secreted. The elaboration of mediators of the inflammatory and immune responses by fibroblasts may initiate and(or) modulate local skin inflammatory reactions and play a protective role in vivo.

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INTRODUCTION

The recent observation by Postlethwaite and Kang (1) that human collagen has chemotactic activity for monocytes suggested that fibroblasts play an important role in recruiting leukocytes to inflammatory-immune sites. However, little attention has been directed at the role of fibroblasts in modulating the normal cellular inflammatory response, or their potential influence in disease states, other than the interest directed at collagen production and fibrosis. In this report, we describe the in vitro elaboration of polymorphonuclear and monocyte chemotactic factors by human fibroblasts. These chemotactic factors are distinct from collagen and the chemotactically active products of complement activation. Another factor is also secreted by fibroblasts that acts on substrates present in human serum to generate the small molecular weight, chemotactically active cleavage product of C5, C5a. These mediators of inflammation may be important in local skin inflammatory and immune events.

METHODS

Fibroblast tissue culture. Dermal fibroblasts obtained commercially (Institute of Medical Research, Camden, N. J.) and from normal healthy volunteers by punch biopsy of the skin were grown in monolayer culture by standard techniques. In a humidified atmosphere which contained 5% CO_2, cultures were maintained in 25 × 10-mm Falcon plastic tissue culture flasks (Falcon Plastics, Div. of Bio Quest, Oxnard, Calif.), containing 5 ml of RPMI 1640 media (Microbiological Associates, Walkersville, Md.) per flask, supplemented with 3% glutamine, penicillin (10,000 μg/ml), streptomycin (10,000 μg/ml), Fungizone (E. R. Squibb & Sons, Princeton, N. J.) (25 μg/ml), and 10% heat-inactivated fetal calf serum. The culture supernatant fluid was removed by pouring off the maintenance media, which were assayed for chemotactic activity. 12 different fibroblast lines were studied over a period of 8 mo. Fibroblasts were passed regularly at a mean interval of 7 d, by pouring off the media, washing the monolayer three times with 5 ml of Hanks' balanced salt solution (National Institutes of Health [NIH] media unit), and then adding 2 ml of trypsin (5 mg/ml, final concentration) to each flask. During

trypsinization, each bottle was agitated for 5 min to facilitate detachment of the cells. Detached cells were collected into 15-ml, capped polystyrene tubes, which contained 5 ml of maintenance media to neutralize the trypsin, and then centrifuged (300 *g* for 10 min at 10°C). The fibroblast pellet was resuspended in 5 ml of maintenance media and placed into the culture flasks.

To exclude contamination of cultures, supernates were regularly tested for the presence of endotoxin with the limulus lysate assay (courtesy of Dr. Ronald Elin, Clinical Pathology, Clinical Center, National Institutes of Health) and were cultured for bacteria, fungi, and viruses. Contaminated cultures were discarded.

Leukocyte chemokinesis and chemotaxis. For most experiments, polymorphonuclear leukocyte (PMN)[1] locomotion was quantitated with the ^{51}Cr radioassay (^{51}Cr, Amersham Corp., Arlington Heights, Ill.) described previously (2). ^{51}Cr-labeled cells were suspended in Gey's balanced salts solution (Microbiological Associates) at a density of 2.3×10^6 PMN/ml and placed in the upper compartment of a modified Boyden chamber that was separated from a lower chamber by two 3-μm micropore cellulose nitrate filters (Sartorius Membranfilter, distributed by Beckman Instruments, Inc., Science Essentials Co., Mountainside, N. J.). Aliquots of fibroblast supernate to be assayed for chemotactic activity were placed in the lower chamber. The chemotactic chambers were incubated at 37°C in 100% humidity and 5% CO_2 for 3 h. It has been shown previously that only PMN migrate into the lower filter during these conditions of incubation, and that the number of migrating cells is proportional to the radioactivity incorporated into the lower filter. After adjustment for variable specific activity and uptake of chromium into the leukocytes, the number of neutrophils in the lower filter was expressed as corrected counts per minute in the lower filter (cor cpm LF). The chemotactic activity in each experimental condition for all experiments was the mean of four replicate determinations. In a few experiments a morphologic assay of PMN chemotaxis, measuring the distance migrated by the leading front of cells, was used (3).

For assessment of monocyte locomotion, human mononuclear cells were isolated from heparinized peripheral blood by the Hypaque- (Winthrop Laboratories, New York) Ficoll (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) technique (4). Chemotactic activity was quantitated by a morphological assay employing a modified Boyden micropore-filter chamber technique and 5-μm Nuclepore polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.) (5). Mononuclear cells were suspended in Gey's balanced salt solution at a density of 3.0×10^6 cells/ml placed in the upper compartment of the chemotactic chamber. After a 90-min incubation at 37°C, 100% humidity, and 5% CO_2, the filters were removed, fixed in triarylmethane and methyl alcohol, and stained with a xanthene and thiazine dye mixture (Harleco, American Hospital Supply Corp., Gibbstown, N. J.) followed by six sequential distilled water rinses. The number of cells that migrated through to the lower surface of the filter were counted in 10 random high power fields as previously described (5).

In some experiments, designed to assess the relative response of neutrophils or eosinophils, a morphologic assay assessing cell locomotion with polycarbonate filters was employed (6).

Standard chemotactic factors employed were sodium caseinate (Difco Laboratories, Detroit, Mich.) (5 mg/ml in 0.85% NaCl), *Escherichia coli* endotoxin 0111:B4 lipopolysaccharide W (Difco Laboratories) -activated serum (7, 8) and the C5 fragment, C5a, partially purified by Sephadex G-75 chromatography of *E. coli* endotoxin-activated serum (9).

Physicochemical properties of chemoattractants. Columns 2.5 cm in diameter and 100 cm in length containing Sephadex G-75 superfine (Pharmacia Fine Chemicals, Inc.) equilibrated with 0.04 M phosphate-buffered saline at pH 7.2, (NIH media unit) were employed. 5 ml of fibroblast-culture supernate were applied to the column, and gel filtration was performed at 4°C with gravity flow and 5-cm^3 fractions were collected. Column effluent protein was monitored for absorbance at 280 nm. Columns were calibrated with proteins of known molecular weight.

Heat stability of chemoattractants present in fibroblast supernate was evaluated by heating an aliquot of the supernate or an active Sephadex fraction to 56°C for 30 min and, on some occasions, to 100°C for 30 min.

To determine whether chemotactic factors present in the crude-fibroblast supernatant fluid were susceptible to proteolytic digestion by trypsin and pronase (Worthington Biochemical Corp., Freehold, N. J.) an aliquot of supernatant fluid was added to both trypsin and pronase so as to result in a 1 and 3% solution of each enzyme, and the mixture was incubated for 2 h at 37°C. Because, as will be shown in Results, the chemoattractant in the fibroblast supernate was heat stable, at the end of 2 h the mixture was heated to 100°C for 10 min to inactivate trypsin and pronase. As controls, aliquots of fibroblast supernate were added to heat-inactivated trypsin and pronase (100°C × 15 min).

In some experiments, fibroblast supernate was treated with *Vibriocholerae* neuraminidase (Worthington Biochemical Corp.); this enzyme preparation released sialic acid from bovine muscillary mucin (Worthington Biochemical Corp.).

The effect of antisera to C3 and antisera to C5 on the chemotactic activity of fibroblast supernates. 50 μl of goat antiserum to the 3rd or 5th human complement components (Meloy Laboratories, Inc., Springfield, Va.) was added to 1-ml aliquots of crude supernatant or chemotactically active fractions obtained by molecular seive chromatography, and the mixture was incubated for 30 min at 37°C, as previously described (8). The aliquots were then tested for chemotactic activity.

Cycloheximide treatment of fibroblast cultures. Cycloheximide was incubated with fibroblast monolayers to study the effect of protein synthesis on elaboration of chemotactic factors into the culture medium. After decanting the culture media, the monolayers were washed three times in Hanks' media and fresh media, containing cycloheximide 20 μg/ml or 50 μg/ml, were added to quadruplicate culture flasks. After 72 h, the supernate was removed, pooled, immediately tested for chemotactic activity and compared with the supernate to which no cycloheximide was added. Additional controls were done in which cycloheximide was added at the end of the culture period to control the supernate immediately before the chemotaxis assay.

Relationship between fibroblast supernatant chemotactic activity and chemotactic activity of collagen preparations. All collagen preparations were the generous gift of Doctors George Martin and Hynda Kleinman (National Institute of Dental Research, National Institutes of Health). Type III collagen was obtained after selective precipitation at 1.0 M NaCl from neutral salt solution of pepsin-solubilized fetal calf skin collagen, as described previously (10). The collagen was reprecipitated twice under the same conditions. The final precipate was dissolved in 0.5 M acetic acid, dialyzed extensively against the same precipitant, and lyophilized.

[1] *Abbreviations used in this paper*: cor cpm LF, corrected counts per minute in lower filter; PMN, polymorphonuclear leukocyte(s).

Type I collagen (chain composition $[\alpha_1(I)_2\alpha_2]$) was obtained from the same solutions of pepsinized, solubilized fetal calf skin collagen by selective precipitation at 2.2 M NaCl. Lathryitic rat skin collagen (type I) was prepared according to the method of Bornstein and Piez (11).

The lyophilized collagen was prepared for chemotaxis studies by dissolving aliquots in 1% acetic acid and stirring overnight (4°C). The acetic acid solutions of collagen were dialyzed extensively against large volumes of 0.04 M phosphate-buffered saline, pH 7.4.

α_1-Chains of collagen were also assayed for chemotactic activity. The constituent chains of type I collagen were separated by CM-cellulose chromatography under denaturing conditions (12).

Assessment of collagen synthesis by fibroblast cultures. Because collagen is precipitated by the addition of 25% ammonium sulphate, the latter was added to an aliquot of either crude-fibroblast supernate or to pooled, Sephadex G-75 fractions which contained chemotactic activity. To enhance the precipitation of collagen, carrier protein in the form of 3% cystallized human albumin (Reheis Co., Inc., Armour Pharmaceutical Co., Kankakee, Ill.) was added to the aliquots tested in some experiments. The clear supernate and the precipitates which were dissolved in 1% acetic acid, and then dialyzed for 24 h against large volumes of 0.04 M phosphate-buffered saline (pH 7.4), were tested for chemotactic activity. In related studies, fibroblast supernatant fluid, obtained from 48-h cultures of fibroblast monolayers, were incubated with collagenase (form III bacterial collagenase, Advanced Biofactures Corp., Lynbrook, N. Y.) for 2 h at 37°C, and the collagen reaction was terminated by heating to 100°C for 10 min.

The amino acid composition of chemotactically active fractions was determined (kindly performed by Dr. Hynda Kleinman, National Institute of Dental Research, National Institutes of Health). For these studies, desalted samples were hydrolyzed in 6N HCl in ampules sealed under N_2 for 24 h at 105°C and analyzed on a Duran auto-analyzer (13). Samples tested included control culture media not exposed to fibroblasts, fibroblast-culture supernate and the chemotactically active fractions from the Sephadex G-75 column.

Effect of ascorbic acid. Established fibroblast monolayers were washed three times with Hanks' balanced salt solution, and fresh culture medium which contained ascorbic acid (50 μg/ml) (Mann Research Laboratories, Inc., New York) was then added. Because Peterkofsky (14) has shown that ascorbic acid decomposes in media, fresh ascorbic acid (50 μg/ml) was added daily. 48 h after the initial removal of supernate, the fibroblast supernate was decanted and tested for chemotactic activity. In control experiments, ascorbic acid was added after the supernate was harvested.

Statistical analysis. Standard errors were used throughout as an estimate of variance, and means were compared by Student's *t* test.

RESULTS

Leukocyte chemotactic activity of fibroblast-culture supernate. When tested by the chemotaxis radioassay, all 12 lines of fibroblasts in tissue culture regularly produced chemotactic activity in the supernate although not all fibroblast lines produced equal activity (Table I). Supernates from three fibroblast lines obtained commercially, that had been stored for variable periods of time, did not produce chemotactic activity after the initial subculture, although chemotactic activity appeared after the second subpassage. The fibroblast lines obtained from skin biopsy performed by us, however, always produced activity after the first subculture. The explanation for this disparity is not known but may relate to conditions of culture or commercial storage of cells. Chemotactic activity of the

TABLE I
Chemotactic Activity of Fibroblast Supernate

Stimulus	Chemotactic activity
	cor cpm LF‡
Buffer (42)*	187±74
Control culture media (18)	297±25
E. coli endotoxin-activated serum (18)	2,004±234
Fibroblast line	
A (28)	1,933±213
B (17)	1,167±169
C (10)	632±199
D (10)	558±132
E (13)	879±162
F (5)	884±195
G (6)	1,436±461
H (4)	1,735±429
I (4)	1,700±465
J (5)	412±64
K (6)	1,237±163
L (3)	479±47
Mean	1,087±226

* Number of separate experiments in parentheses.
‡ Mean±SEM.

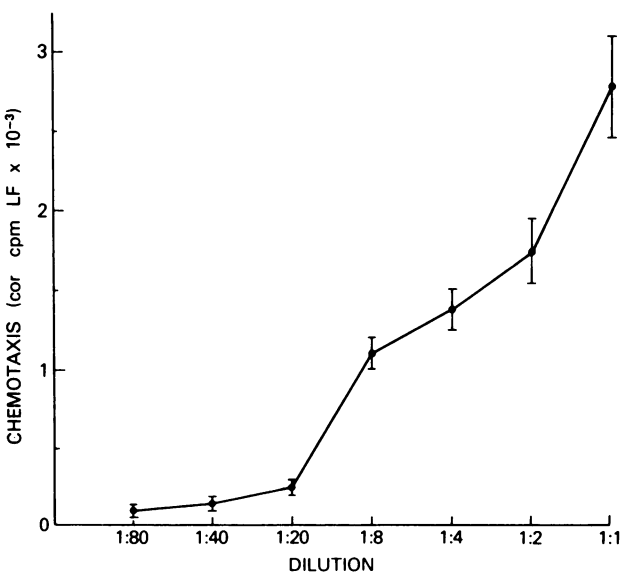

FIGURE 1 Chemotactic activity of fibroblast-culture supernate (dose-response). The effect of various concentrations of fibroblast-culture media on the PMN chemotactic response with the ^{51}Cr radioassay of chemotaxis. Data are the mean ±SEM of four determinations.

TABLE II

*PMN Chemokinetic and Chemotactic Activity of Fibroblast Supernates**

Concentration of supernate in lower compartment, %	Concentration of supernate in upper compartment (%)				
	0	25	50	75	100
	cor cpm LF				
0	174±357*	171±312	168±13	112±18	76±5
25	733±202	350±54	208±17	103±14	98±9
50	1,612±242	660±75	401±230	218±34	114±23
75	1,606±205	777±93	436±41	422±47	122±17
100	2,126±659	845±116	579±45	143±41	63±52

* PMN locomotion assayed with radioassay with data expressed as cor cpm LF (mean±SEM, four determinations). Fibroblast supernate was added in the indicated concentrations to the lower (stimulus) or upper (cell) compartment of the chemotactic chambers.

supernate was comparable in most lines to that of sodium caseinate (5 mg/ml) or *E. coli* endotoxin-activated serum (5%). Fig. 1 demonstrates a chemotactic dose-response relationship, with significant activity detected through a 1:10 dilution of the crude-fibroblast supernate.

To assess the chemokinetic and chemotactic properties of the fibroblast supernate, culture fluid was added in varying concentrations to both the lower compartment (stimulus side), as well as the upper compartment (cell side) of the chemotactic chambers (Table II). With progressive increase in the concentration of the fibroblast supernate in the lower chamber, migration of leukocytes into the lower filter was enhanced as demonstrated in the vertical columns of Table II. Similarly, when the concentration gradient was eliminated by adding higher concentrations of chemoattractant to the upper chamber, inhibition of chemotaxis was observed (horizontal columns of Table II). In the absence of a chemical gradient, but with increasing concentrations of the chemotactic stimulus in both chambers, migration was increased through a concentration of 75% the original supernatant concentration (diagonal column in Table II). The decreased leukocyte migration, which occurred when 100% concentrations of fibroblast supernate were present in both chambers, probably reflects leukocyte "deactivation" from high concentrations of the chemotactic factor. These studies demonstrate that crude-fibroblast supernate has both chemokinetic and chemotactic properties.

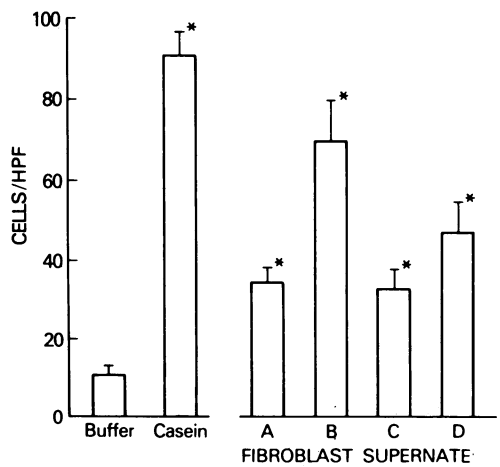

FIGURE 2 Chemotactic response of human monocytes to four different preparations of fibroblast-culture media with a morphologic assay. The response to buffer or sodium caseinate (5 mg/ml) is shown in the left panel for reference. Each bar represents the mean±SEM, four determinations. *$P < 0.01$ vs. buffer controls. HPF, high power field.

In studies of monocyte locomotion, the fibroblast supernate obtained from each of four fibroblast lines was a chemotactic stimulus for monocytes ($P < 0.01$ vs. buffer) (Fig. 2). In studies similar to those shown in Table II, the crude-fibroblast supernate had chemokinetic and chemotactic activity for monocytes (data not shown).

Culture conditions for optimal release of chemotactic factor. In initial experiments, chemotactic activity of freshly divided cells was assessed over time to determine what effect fibroblast replication and(or) contact inhibition had on the elaboration of chemotactic factors. Chemotactic activity was not detected in the fibroblast supernates during the first 48 h of growth, and activity did not appear until a monolayer of fibroblasts was visually noted in the culture flasks. To assess whether the production of the chemotactic activity

was merely dependent upon fibroblast density or influenced by another parameter, such as contact inhibition during monolayer formation, additional studies were performed in which fibroblasts were either maintained in monolayer or placed in equivalent cell number in fresh flasks and permitted to multiply. Volume of culture fluid was equivalent under both conditions. As seen in Fig. 3, significantly more chemotactic activity was present in days 2–5 in the culture fluid of fibroblasts maintained in the stationary phase compared with fibroblasts in the multiplication phase ($P < 0.05$). However, after 6 d, when maximal activity was reached, no significant difference in the activity was noted.

The effect of continued subpassage was studied in four fibroblast lines actively producing chemotactic activity (Fig. 4). The chemotactic activity of four fibroblast lines decreased with subcultures, with significantly less chemotactic activity noted by the sixth subculture ($P < 0.01$). Diminished chemotactic responses continued throughout additional subcultures.

Although the culture medium possessed no intrinsic PMN chemotactic activity, it was necessary to assess whether the chemotactic activity in the fibroblast supernate after culture was related to a chemotactic factor released by fibroblasts, or whether it represented the effect of a secreted product, such as a proteolytic enzyme, which generated a chemotactic factor from a substate present in the fetal calf serum included in the culture media. Accordingly, fibroblasts were cultured in media free of fetal calf serum for 6 d in both the stationary and multiplication phase. Under each condition chemotactic activity was present in the culture supernate (1,828±102 cor cpm LF for fibroblast supernate compared with 180±18 cor cpm LF for cell-free controls, $P < 0.001$). In additional experiments, 0.1-ml aliquots of fibroblast supernate were added to 0.1 ml fresh fetal calf serum or heat-inactivated fetal calf serum in 0.8 ml of culture media and incubated for 1 h at 37°C. No chemotactic activity was generated from fresh fetal calf serum or heat-inactivated fetal calf

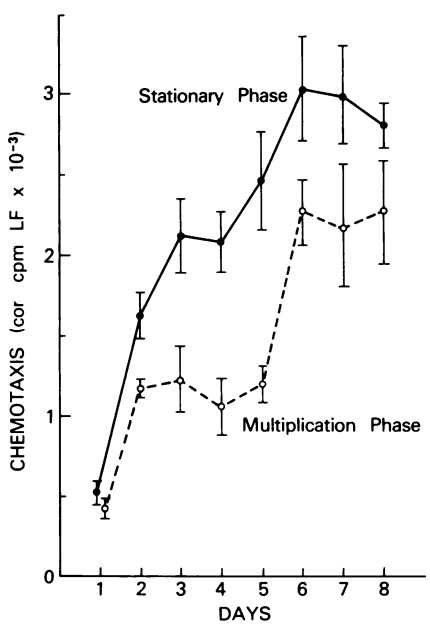

FIGURE 3 Production of chemotactic activity by fibroblast cultures. The elaboration of PMN chemotactic activity by fibroblasts in the stationary or multiplication phases at the indicated time of subculture. Chemotaxis was measured with the ^{51}Cr radioassay, and each point represents the mean±SEM of four determinations.

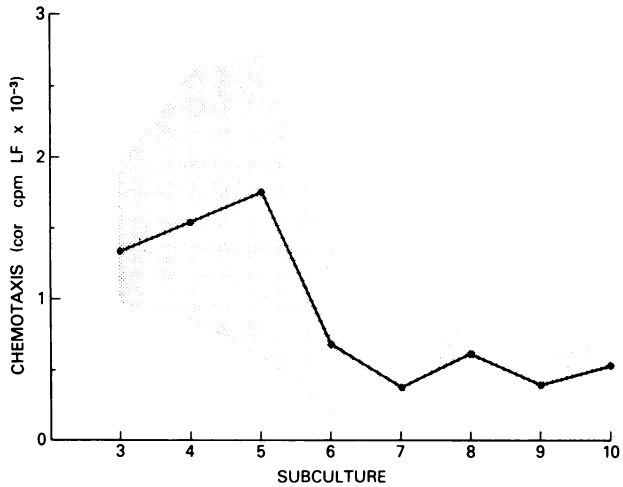

FIGURE 4 Effect of repeated subcultures of fibroblasts on chemotactic factor production. The effect of subculture on the ability of four fibroblast lines to produce PMN chemotactic activity as assessed with the ^{51}Cr radioassay. Each point represents the mean chemotactic activity of four different fibroblast lines, and the shaded area indicates the range of the response.

TABLE III
Cycloheximide Inhibition of PMN Chemotactic-Factor Elaboration by Fibroblasts in Tissue Culture

Stimulus	Chemotactic activity	P*
	cor cpm LF	
Fibroblast supernate‡	1,609±112§	—
+ Cycloheximide before culture		
20 μg/ml	482±87	<0.01
50 μg/ml	219±45	<0.01
+ Cycloheximide after culture		
20 μg/ml	1,713±17	>0.05
50 μg/ml	1,660±132	>0.05

* Significance level of difference from fibroblast supernate, Student's *t* test.
‡ Fibroblast cultures had indicated amount of cycloheximide added either before or after a 72-h culture.
§ PMN in lower filter, mean±SEM, four determinations.

serum. Thus, the chemotactic activity in the crude-fibroblast supernate cannot be attributed to a product of a fibroblast factor interacting with a substate in the heat-inactivated fetal calf serum included in the culture media.

Requirement of protein synthesis for elaboration of chemotactic factor. The chemoattractant present in fibroblast supernates was not detected in freeze-thaw homogenates of fibroblasts, which suggests that the chemotactic factor was not preformed. In related studies, designed to assess the requirement of protein synthesis for fibroblast elaboration to chemotactic factor, fibroblast monolayers were cultured in the presence of cycloheximide. As shown in Table III, concentrations of cyloheximide which are known to inhibit protein synthesis (20 μg/ml) significantly decreased the chemotactic activity of the fibroblast supernate. In control experiments in which cycloheximide was added to the fibroblast supernate just before adding to the chemotactic chambers, no inhibition of chemotaxis was observed.

Preliminary characterization of the chemotactic activity in fibroblast supernates. The chemotactic activity of the crude-fibroblast supernate was heat stable at 56°C in all 12 fibroblast lines shown in Table I, and, in addition, in three cell lines further examined, was stable when heated at 100°C for 30 min. Crude supernate incubated at 37°C for a period of 2 mo under sterile conditions retained its functional activity.

The fibroblast supernatant PMN chemotactic factor was susceptible to digestion by both trypsin and pronase (Table IV). Treatment of supernate with trypsin (10 mg/ml) inhibited the chemotactic activity by 75%, although incubation with pronase (10 mg/ml) inhibited the activity by 85%. Addition of heat-inactivated trypsin or pronase had no effect on the chemotactic activity of fibroblast supernates. In other experiments, designed to assess the requirement of sialic acid and intact glycoprotein structure, neuraminidase was incubated with fibroblast supernates as described (15). As shown in Table IV, neuraminidase did not influence the chemotactic activity of the fibroblast supernate.

Fractionation of fibroblast supernates by gel filtration. Unconcentrated crude-culture supernates obtained from 5-d-old cultures of fibroblast monolayers were fractionated by Sephadex G-75 chromatography (Fig. 5). The chemotactic activity eluted in two major regions with estimated molecular weights >100,000 and <10,000. These fractions corresponded to fractions containing the majority of protein, as estimated by absorbance at 280 nm. Fractions containing the 100,000 and 10,000 materials were pooled. In contrast to the heat stability of the chemotactic activity of crude supernate, the chemotactic activity present in each of the fraction pools from the Sephadex G-75 column were heat labile with the chemotactic activity of the large

TABLE IV

Effect of Trypsin, Pronase, and Neuraminidase on PMN Chemotactic Activity on Fibroblast Supernate

Substance added	Chemotactic activity*	P‡
	cor cpm LF	
None	2,124±140	—
Trypsin (10 mg/ml)	610±114	<0.01
Heat-inactivated trypsin	1,870±152	>0.05
Pronase (10 mg/ml)	500±78	<0.01
Heat-inactivated pronase	1,987±167	>0.05
Neuraminidase (5 μg/ml)	1,565±75	>0.05
Heat-inactivated neuraminidase	1,874±172	>0.05

* ^{51}Cr-labeled PMN in lower filter, mean±SEM, four determinations.

‡ Significance of difference from untreated supernate (Student's *t* test).

molecular weight material reduced by heating 30 min at 56°C from 740±65 to 152±37, and the activity in small molecular weight peak from 859±82 to 192±25 cor cpm LF ($P < 0.01$ for each comparison). In three experiments, incubation of the Sephadex G-75 fractions with goat antisera to the third and fifth complement components did not inhibit the chemotactic activity of either of the two pooled fractions obtained by Sephadex

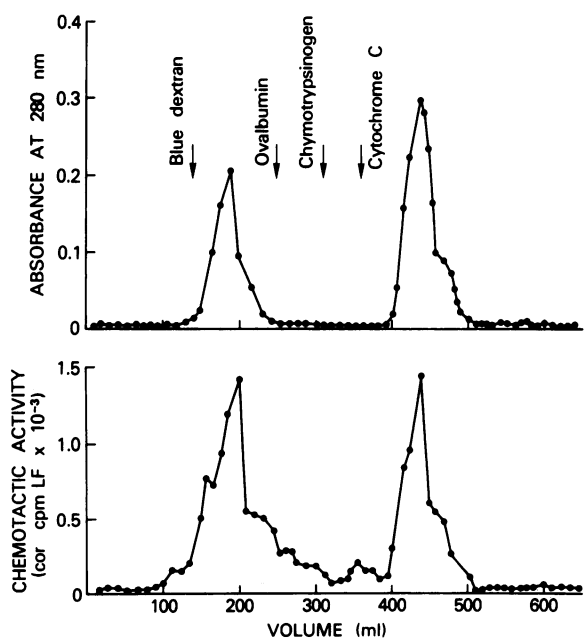

FIGURE 5 Sephadex G-75 chromatography. The elution profile from Sephadex G-75 of fibroblast-culture fluid. Protein is shown in the upper panel (OD 280 nm), and the elution of standard proteins is shown. PMN chemotactic activity in the column fractions, depicted on the origin as cor cpm LF, is shown in the lower panel.

chromatography (i.e., the mol wt 100,000 material had a mean control chemotactic activity of 1,384±172 cor cpm LF which was 1,722±209 and 1,743±307 after treatment with anti-C5 and anti-C3, respectively; and the fraction less than mol wt 10,000 had control chemotactic activity of 1,401±195 which was 1,209±294 and 1,527±294 after treatment with anti-C5 and anti-C3). In control experiments, goat antisera to C5, but not C3, inhibited the chemotactic activity of a partially purified preparation of C5a (from 1,640±170 to 430±47 cor cpm LF).

In related studies it was shown that the fractions obtained from the Sephadex G-75 column, which were chemotactic for neutrophils, also attracted monocytes. With the morphologic assay of monocyte locomotion, the mean monocyte response with the mol wt 100,000 material was 43±4 monocytes per high-power field vs. buffer in which 11 cells per high-power field migrated (three experiments, $P < 0.01$). The monocyte response to the small molecular weight material was 31±5 cells per high-power field ($P < 0.01$ vs. buffer). In other studies assessing leukocyte migration with the polycarbonate filter technique with PMN from a patient with 30% eosinophilia, no preferential activity of eosinophils over neutrophils were detected with either the crude-culture fluid or fractions obtained from the Sephadex G-75 chromatography.

Relationship of chemotactic factors in fibroblast-culture fluid to collagen. Fibroblasts produce collagen which is known to be a chemotactic factor for monocytes but not neutrophils (16). The following studies were designed to assess the relationship of the chemotactic activity we detected in fibroblast-culture fluid to collagen.

To initially address this question, we reassessed the chemotactic properties of several collagen preparations. Type I collagen of lathrytic rat skin origin as well as α_1-chains of rat skin collagen were assayed at three different concentrations for human neutrophil and monocyte chemotactic activity. As shown in Table V, whereas both the type I collagen and α_1-chain preparations stimulated monocyte migration, they caused no or feeble stimulation of neutrophil migration. Similarly, pepsin-treated calf skin type III collagen was not chemotactic for neutrophils.

The chemotactic response of monocytes to collagen was concentration-dependent (Fig. 6). Native collagen was a more potent chemotactic stimulus than α_1-chains. Type III collagen in similar doses to type I collagen did not stimulate either monocyte or neutrophil migration.

Addition of ascorbic acid to human skin fibroblast culture is known to increase the synthesis and secretion of collagen, most of which is found in the medium as procollagen (14). Supernate of fibroblast cultures were assayed for chemotactic activity 24, 48, and 72 h after the addition of ascorbate. Ascorbate failed to increase the chemotactic activity of the fibroblast supernate compared with control experiments in which ascorbate was added just before the chemotaxis assay (data not shown).

TABLE V
Chemotactic Response of Monocytes and PMN to Collagen and α_1-Chains

Substance tested	Concentration	Chemotactic response*	
		Monocytes	PMN
	mM	*cells/HPF‡*	*cor cpm LF*
Buffer	—	12±2	190±25
Fibroblast supernate	—	25±2§	1,904±108§
Rat skin collagen			
Type I	1.0	49±8§	173±53
	0.3	35±7§	250±25
	0.1	29±4	72±10
α_1-Chain	2.7	26±3§	87±13
	0.9	20±2	65±6
Pepsin-treated calf skin collagen			
Type I	2.0	41±3§	121±17
	1.0	45±5§	260±32
Type III	1.3	13±3	165±22
	0.6	11±2	212±29

* Monocyte chemotaxis with a morphologic assay, and PMN chemotaxis with a ^{51}Cr radioassay (mean±SEM, four determinations).
‡ HPF, high power field.
§ $P < 0.01$ vs. buffer, Student's t test.

Treatment of fibroblast-culture media with col-

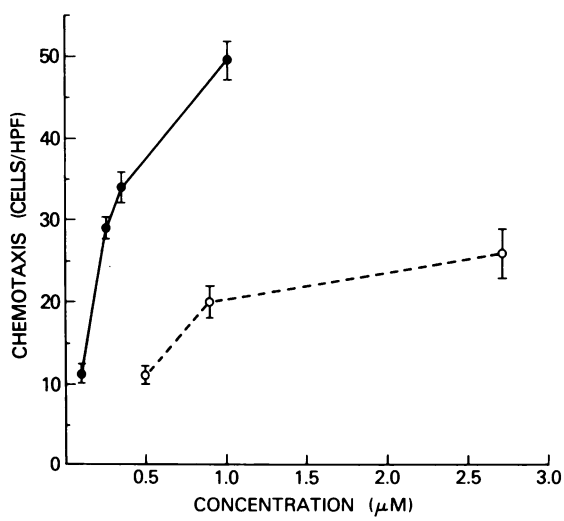

FIGURE 6 Chemotactic response of human monocytes to the indicated concentrations of rat skin collagen (●---●) and the α_1-chain (○---○) of rat skin with a morphologic assay of chemotaxis. Each point represents the mean±SEM of four determinations. HPF, high power field.

lagenase as described in Methods had no significant effect on PMN or monocyte chemotactic activity (Table VI). In other experiments, the monocyte and PMN chemotactic activity present in serum-free fibroblast-culture media and in Sephadex G-75 fractions was not precipitated by 25% ammonium sulfate. In further studies, amino acid analysis of the Sephadex G-75 fractions with chemotactic activity failed to detect amino acids, such as hydroxyproline and hydroxylysine, characteristic of collagen. Similarly, six different crude-fibroblast supernates which had marked chemotactic activity were analyzed, and no amino acids characteristic of collagen were detected. The latter finding may, however, be the result of large amounts of protein present in the supernatant fluid (fetal calf serum) obscuring the presence of collagenous proteins.

Generation of chemotactic activity from human serum by fibroblast supernates. Although incubation of human fibroblast supernates with fetal calf serum did not generate chemotactic activity, this was not the case with human serum. Incubation of fibroblast supernates with 10% human serum generated chemotactic activity (Fig. 7) which could not be explained by the simple additive chemotactic effect of the fibroblast supernate with the *de novo* activity of serum; i.e., the concentration of fibroblast supernate was below that stimulating chemotaxis (Fig. 1). Six fibroblast lines, tested on three occasions, generated PMN chemotactic activity from human sera. The activity produced was heat stable (56°C for 30 min) and inhibited by goat antisera to human C5 (from 1,750±320 to 374±112 cor cpm LF, $P < 0.01$) but not C3. Preheating the human serum at 56°C for 30 min before the addition of the fibroblast supernate prevented serum activation. The serum activator present in the fibroblast supernate was heat resistant (56°C for 30 min). Fractionation of human serum that had been activated with fibroblast supernate on Sephadex G-75 indicated the chemotactically active material eluted with an estimated molecular weight of 12,500 was resistant to heating 30 min at 56°C and was inactivated by goat antisera to human C5 but not C3.

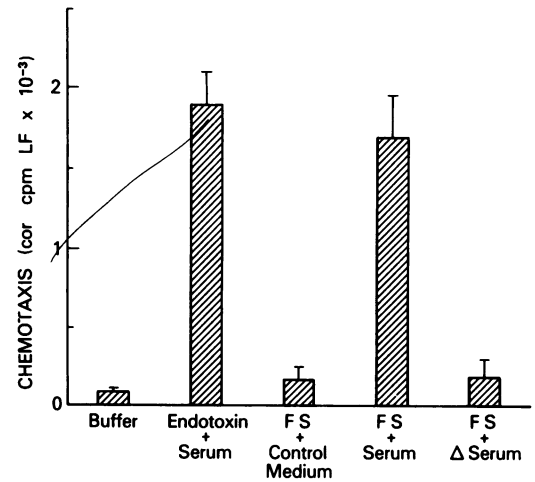

FIGURE 7 Generation of chemotactic activity by fibroblast supernate. The ability of fibroblast supernate (FS) to generate PMN chemotactic activity from human serum (10% FS vol/vol in serum). Each bar represents the number of PMN migrating into the lower filter with the ^{51}Cr radioassay of chemotaxis (mean±SEM, four determinations). The response of buffer or *E. coli* endotoxin-activated serum is shown for reference, and Δ serum represents serum heated to 56°C × 30 min before the addition of fibroblast supernate.

TABLE VI
*Effect of Collagenase on the Chemotactic Activity of Fibroblast-Culture Media**

Condition	Chemotactic response‡	
	Monocytes	PMN
	cell/HPF	*μm migrated*
Buffer	10±2	65±5
Fibroblast media + buffer	72±6	120±4
Fibroblast media + collagenase§	62±7	114±5
Fibroblast media + boiled collagenase§	67±5	111±6

* Serum-free culture media obtained from monolayers of fibroblasts incubated 48 h with RPMI-culture media.
‡ Monocyte chemotaxis quantitated as cells per high power field (HPF) migrating to the lower surface of a polycarbonate filter; PMN chemotaxis quantitated as distance migrated by the leading front of a PMN population into a cellulose nitrate filter. Mean±SEM, three determinations.
§ Each cubed centimeter of fibroblast-culture media was treated with sufficient collagenase to digest 70 μg of collagen. For controls, collagenase was boiled for 10 min before addition. Collagenase treatment was for 2 h at 37°C, at which time the reaction was terminated by boiling (10 min).

DISCUSSION

Although the primary function of fibroblasts appears to be the production of collagen and mucopolysaccharides, recently fibroblasts have been shown to secrete a number of other biologically active products including Clq, interferon, and migration inhibitory factor (17, 18). The demonstration of receptors for C3 on fibroblasts, as well as increased fibroblast proliferation, locomotion, and collagen secretion (19–21) in response to lymphokines suggests that the fibroblast is well suited to actively participate in inflammatory and immune reactions mediated by different etiological mechanisms.

All 12 fibroblast lines we studied produced factors which directly stimulated neutrophil and monocyte random locomotion and chemotaxis, although the

chemotactic activity of the different lines was not always comparable. The reason for this is unclear, but may, in part, be related to the number of consecutive subcultures.

The chemotactic factor in the fibroblast-culture fluid is protein in nature, being heat stable and sensitive to pronase and trypsin digestion. Two active fractions were obtained by Sephadex G-75 chromatography, both of which were, in turn, heat sensitive. It is remarkable that the chemotactic activity in crude supernate (but not the Sephadex fractions) was resistant to boiling for 30 min.

Collagen has been traditionally considered a structural protein intimately involved in tissue repair and remodelling and more recently has been thought to play a role in the inflammatory and immune response, particularly in view of its chemotactic activity for monocytes (1). In this study we have confirmed the observation of Postlethwaite and Kang (1) that type I collagen and its constituent chains are chemotactic for monocytes. The failure to demonstrate that collagen derived from skin has potent chemotactic activity, for neutrophils is in keeping with the original observation of Stecher (22) but conflicts with the results of Chang and Houck (16) who showed that rat collagen was chemotactic for rat neutrophils in vivo. Type III collagen, which is composed of α_1- (III) chains and is found in young skin and blood vessels, was not chemotactic for either monocytes or neutrophils, although the chemotactic factors present in fibroblast culture medium were chemotactic for both.

The chemoattractants we detected in fibroblast cultures can be distinguished from collagen. Fibroblasts cultured under conditions we used produce much less collagen than are required to demonstrate monocyte chemotactic activity with pure collagen preparations. The fibroblast products were not inactivated by collagenase and, in addition, unlike collagen the chemoattractants present in our fibroblast supernates were not precipitated by 25% ammonium sulphate. Moreover, ascorbic acid, a stimulus of fibroblast collagen production (23), did not stimulate elaboration of chemotactic factors. No trace of characteristic collagen amino acids such as hydroxyproline and hydroxylysine were detected on amino acid analysis of the chemotactially active fractions obtained from Sephadex G-75 chemotography. Finally, the chemotactically active fractions isolated from the Sephadex columns were sensitive to pronase and trypsin whereas type I collagen is resistant to these enzymes (23).

In addition to *de novo* chemotactic activity, the fibroblast-culture fluid was shown to be capable of generating chemotactic activity from human serum which, based on its heat stability, molecular weight, and inactivation to goat antisera to human C5 but not C3, probably represents the chemotactically active C5 fragment C5a. This latter property is not unexpected as other mammalian cells contain enzymes (proteases) that can cleave C5a from the 5th component of complement (24–26). Hurley first showed that incubation of disrupted normal tissue incubated with serum resulted in generation of neutrophil chemotactic activity (27). Similarly, infection of tissue cultures with different viruses results in the release of a tissue factor which generates a chemotactic product from C5, and, likewise, lysates of infected cultures produce chemotactic activity by a similar mechanism (28, 29); it is noted there was no evidence of bacterial or viral infection of our fibroblast cultures. The possibility that the chemotactic-generating activity we detected in the fibroblast-culture media is stored in fibroblast granules and secreted into the extracellular melieu, as has been shown in neutrophils (26), is intriguing and worthy of additional study.

Thus the data in this paper show that human fibroblasts can synthesize chemotactic factors distinct from collagen which are active for both neutrophils and monocytes. Fibroblasts, therefore, have the potential to secrete chemotactic factors which recruit PMN and mononuclear leukocytes depending upon local tissue needs.

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