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Chemotactic attraction of human fibroblasts to type I, II, and III collagens and collagen-derived peptides

(tissue injury/collagenase/fibroblast/chemotaxis inflammation)

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ABSTRACT **The chemotactic response of human dermal fibroblasts to type I, II, and III human collagens and collagen-derived peptides was quantitated by an *in vitro* assay. All three native human collagens and constituent α chains can serve as chemoattractants for fibroblasts *in vitro*. When type I, II, and III collagens were digested by bacterial collagenase, the resulting peptides were also chemotactic. In addition, synthetic di- and tripeptides containing hydroxyproline were also chemotactic for fibroblasts. Since collagen is degraded and remodeled at sites of tissue injury and inflammation, these findings suggest that collagen and collagen-degradation peptides might function as chemotactic stimuli for fibroblasts *in vivo* and attract these cells to effect repair of damaged tissue.**

Fibroblasts are found in inflammatory lesions resulting from tissue injury by a variety of different agents. They effect repair of damaged tissue by synthesizing and laying down extracellular components which constitute the scar. The mechanisms whereby these specialized effector cells are attracted to sites of tissue injury and inflammation are poorly understood.

Collagen is the most abundant and ubiquitous connective tissue protein. So far, at least four distinct types of collagen have been found. Type I, II, and III collagens and their constituent α chains have been well characterized (1–12). Type IV collagen, isolated initially from basement membranes, has been more difficult than the other three collagens to characterize biochemically and may be heterogeneous (13–15).

We have recently developed a method by which fibroblast chemotaxis to various stimuli can be quantitated *in vitro* (16). By using this technique, we have isolated and characterized a human lymphocyte-derived chemotactic factor for fibroblasts, LDCF-F (16). In the present study we have found that type I, II, and III collagens and collagen-derived peptides can effect chemotactic migration of fibroblasts.

MATERIALS AND METHODS

Preparation of Collagens and Constituent α Chains. Human type I and II collagens were obtained from the bone and cartilage of extremities previously amputated to treat peripheral vascular disease. Type III collagen was purified from human livers obtained at autopsy. Collagens were solubilized from these tissues by limited proteolysis with pepsin under nondenaturing conditions and purified by methods previously described (3, 9). Type I collagen was also obtained from the skin of 3-week-old lathyritic chickens (17).

Heat-denatured collagen solutions were fractionated on carboxymethyl-cellulose to isolate purified α chains as described (1, 3, 9).

Fibroblast Chemotaxis Assay. Fibroblast chemotaxis was measured *in vitro* by methods previously described in detail (16). The assay uses blindwell modified Boyden chemotaxis chambers equipped with gelatin-treated polycarbonate filters having 8-μm pores. Normal human dermal fibroblasts maintained as continuous cell lines were used as indicator cells in the assay. Fibroblasts were harvested from monolayers by either trypsinization or EDTA treatment. Fibroblasts chemotactic activity was determined after chambers were incubated for 150 min by counting the fibroblasts migrating to the lower surface of the polycarbonate filters in 20 oil immersion fields. Substances were assayed in triplicate, and the final chemotactic activity of a sample was expressed as the mean ± SEM.

Lyophilized collagen and α chain preparations were solubilized for use in the chemotaxis studies by dissolving them in 0.5 M acetic acid, stirring them overnight (4°), and then dialyzing them against large volumes of 10 mM phosphate/0.14 M NaCl at pH 7.4 for 24 hr and subsequently against 10 mM glycylglycine/0.14 M NaCl at pH 7.2 for 24 hr at 4°.

Degradation of α Chains by Cyanogen Bromide and Pepsin. α Chains obtained from lathyritic chick skin collagen were cleaved by incubation with cyanogen bromide (CNBr) (18, 19). In different experiments, α chains (5 mg each) from lathyritic chick skin collagen were separately incubated at 37° in 1 ml of 10 mM HCl with pepsin (50 μg). The lyophilized CNBr- and pepsin-cleaved α chains were prepared for analysis of chemotactic activity for fibroblasts by separately dialyzing them with glycylglycine/NaCl in an ultrafiltration cell containing a UM 2 membrane (Amicon Corp., Lexington, MA).

Collagen Degradation by Collagenase. Solubilized type I, II, and III human collagens (6 mg in 2.3 ml of glycylglycine/NaCl containing 1 mM $CaCl_2$) were each digested for 18 hr at 37° with purified bacterial collagenase (60 μg), (CLSPA, Worthington Biochemical Corp., Freehold, NJ) and assayed for fibroblast chemotactic activity.

Hydroxyproline Assay. The amount of collagen or collagen peptides contained in dialyzed samples was calculated from hydroxyproline content of assayed aliquots (20).

Gel Filtration of Collagenase-Degraded Collagen. Peptides generated by bacterial collagenase digestion of type I, II, and III human collagens were separately fractionated on a 1.5 × 35-cm column of Sephadex G-50 equilibrated with glycylglycine/NaCl.

Noncollagenous Proteins. Bovine serum albumin and ovalbumin (Sigma Chemical Co., St. Louis, MO) were dissolved in saline. Aliquots of these solutions were adjusted to pH 2.0 by the addition of 6 M HCl, digested at 37° with pepsin (substrate to enzyme ratio of 100:1, wt/wt) for 6 hr, and flash evaporated.

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Abbreviations: LDCF-F, lymphocyte-derived chemotactic factor for fibroblasts; glycylglycine/NaCl, 10 mM glycylglycine/0.14 M NaCl at pH 7.2.

Proteolysis was documented by the manual ninhydrin reaction on sample aliquots (21).

Tri- and Dipeptides. Synthetic tri- and dipeptides containing glycine, proline, or hydroxyproline were obtained from Sigma Chemical Co., St. Louis, MO. Peptides were each dissolved in glycylglycine/NaCl, the pH was adjusted to 7.2, and the samples were then assayed for fibroblast chemotactic activity.

Amino Acids. L-Lysine, L-proline, L-tryptophan, DL-4-hydroxyproline (Calbiochem, San Diego, CA), DL-allohydroxylysine (Schwarz/Mann, Orangeburg, NY), L-glycine, and L-glutamine (Sigma Chemical Co., St. Louis, MO) were dissolved in glycylglycine/NaCl and assayed for fibroblast chemotactic activity.

RESULTS

Collagen and α Chains as Chemoattractants. Human type I, II, and III collagens and constituent α chains induced fibroblast migration in modified Boyden chambers (Fig. 1). Native type I, II, and III collagens were of similar potency (Fig. 1). Purified α chains from these three collagens were less potent than the native collagen molecules from which they were derived (Fig. 1).

It was essential to determine whether the mere contact of collagen with fibroblasts was causing them to migrate through the filter pores or whether they were migrating in response to a concentration gradient of collagen, established by placing collagen exclusively in the lower compartment of the chemotaxis chamber. In order to resolve this issue, type I, II, and III collagens were each added to a final concentration of 0.4 μM to the upper and lower compartments of chemotaxis chambers in various combinations with each other, with buffer (glycylglycine/NaCl), and with supernatant from antigen-stimulated lymphocyte culture rich in LDCF-F (Table 1). When buffer or LDCF-F was added to fibroblasts in the upper chamber compartment, there was migration of fibroblasts to each of the three types of collagen (Table 1). When type I, II, or III collagen was added to fibroblasts in the upper chamber compartment there was no migration to each of the three types of collagen when these were added to the lower chamber compartment, but there was migration when LDCF-F was added to the lower compartment (Table 1).

These data indicate that fibroblast migration is toward the source of a gradient of type I, II, and III collagens and LDCF-F and suggest that these substances induce chemotaxis of fibroblasts. These data also suggest that type I, II, and III collagens probably share a common receptor for chemotaxis on the surface of the fibroblast, but that a different receptor is present for LDCF-F.

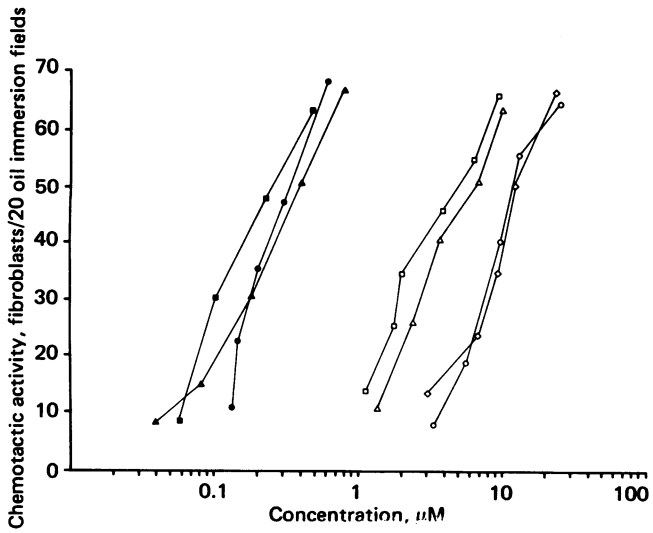

FIG. 1. Chemotactic response of human dermal fibroblasts to human type I (●), type II (▲), and type III (■) collagens and to α chains α1(I) (○), α2(I) (◇), α1(II) (△), α1(III) (□).

Table 1. Effect of concentration gradient of chemoattractants on fibroblast migration

Upper compartment*	Lower compartment*	Cell migration, fibroblasts/20 OIF† (mean ± SEM)
Buffer	Type I collagen	44 ± 5
	Type II collagen	50 ± 7
	Type III collagen	52 ± 6
	LDCF-F	56 ± 5
	Buffer	5 ± 1
Type I collagen	Type I collagen	6 ± 2
	Type II collagen	6 ± 2
	Type III collagen	5 ± 1
	LDCF-F	46 ± 6
	Buffer	5 ± 1
Type II collagen	Type I collagen	7 ± 2
	Type II collagen	8 ± 1
	Type III collagen	5 ± 1
	LDCF-F	49 ± 4
	Buffer	5 ± 1
Type III collagen	Type I collagen	7 ± 1
	Type II collagen	5 ± 1
	Type III collagen	8 ± 2
	LDCF-F	43 ± 5
	Buffer	7 ± 2
LDCF-F	Type I collagen	51 ± 7
	Type II collagen	46 ± 4
	Type III collagen	53 ± 6
	LDCF-F	6 ± 2
	Buffer	4 ± 1

* Glycylglycine/NaCl, type I, II, or III human collagens (final concentration 0.4 μM), or supernatant from a 96-hr culture of human peripheral blood lymphocytes stimulated with streptokinase-streptodornase (1) rich in LDCF-F (final concentration 10% vol/vol) were added to the upper and/or lower compartments of the chemotaxis chambers. After a 150-min incubation, fibroblast migration to the lower surface of the filters was quantitated.

† Oil immersion fields.

We have also found that human dermal fibroblasts recognize chick type I collagen and α chains as chemotactic stimuli (see Tables 2 and 3). It appears, therefore, that the fibroblast chemotactic response to collagen is not species specific.

Inhibition of Collagen-Induced Chemotaxis by α1(I). It was of interest to determine whether α1(I) chain from chick skin collagen would inhibit the migration of fibroblasts to native collagens. To answer this question, we preincubated fibroblasts at 37° for 30 min with 10 μM α1(I) or an equal volume of buffer (glycylglycine/NaCl), washed them once with medium, and tested them for their ability to migrate in the chemotaxis assay to the native collagens, α1(I), and LDCF-F. Preincubation of fibroblasts with α(I) caused their migration to type I, II, and III human collagens and to α1(I) to be inhibited (Table 2). However, migration of fibroblasts to LDCF-F was unaffected by preincubation with α1(I) (Table 2).

Chemotactic Response of Different Cell Lines to Collagen. Six different human dermal fibroblast lines were tested for their ability to repond to aliquots of the same collagen preparation. All six cell lines migrated in response to lathyritic chick skin collagen ($P < 0.0005$ by the paired Student's t test), suggesting

Table 2. Inhibition of collagen-induced chemotaxis by α1(I)

Substance tested*	Fibroblasts preincubated with†	Chemotactic activity, fibroblasts/20 OIF‡ (mean ± SEM)
Type I collagen (0.4 μM)	Buffer	57 ± 5
	α1(I)	9 ± 2
Type II collagen (0.4 μM)	Buffer	63 ± 3
	α1(I)	7 ± 1
Type III collagen (0.4 μM)	Buffer	51 ± 3
	α1(I)	11 ± 1
α1(I) (3.0 μM)	Buffer	44 ± 4
	α1(I)	4 ± 1
LDCF-F (10%, vol/vol)	Buffer	62 ± 7
	α1(I)	57 ± 6
Buffer§	Buffer	3 ± 1
	α1(I)	4 ± 1

* Type I, II, and III human collagens, α1(I) from chick collagen, and LDCF-F obtained as described in Table 1 were tested for their ability to induce migration of fibroblasts that had been preincubated with α1(I) or buffer.

† Fibroblasts harvested by trypsinization from monolayer cultures were washed once with serum-supplemented Eagle's minimal essential medium and once with serum-free Eagle's medium. Washed fibroblasts were preincubated at 37° for 30 min with Eagle's medium containing 10 μM α1(I) from chick skin collagen or an equal volume of buffer (glycylglycine/NaCl). After incubation, fibroblasts were washed with Eagle's medium and then used in the chemotaxis assay.

‡ Oil immersion fields.

§ Glycylglycine/NaCl.

that dermal fibroblasts in general recognize collagen as a chemoattractant (Table 3).

Fibroblasts Harvested with EDTA. The use of trypsin to harvest fibroblasts from monolayer cultures could theoretically alter the plasma membrane and expose an artificial receptor for collagen. When fibroblasts were dispersed with 5 mM EDTA, they were able to respond chemotactically to lathyritic chick skin collagen and the α chains of chick skin collagen (data omitted). These data suggest that a receptor(s) for collagen and α chains is normally present and accessible on fibroblast plasma membranes.

Collagen-Derived Peptides. In order to determine whether the large size of intact α chains (molecular weight 94,000) was essential for chemotaxis, or whether fibroblasts could respond to smaller fragments of collagen, α chains of type I lathyritic chick skin collagen were degraded separately by CNBr and pepsin, and the products were tested for fibroblast chemotactic activity. Peptides generated by degrading α1 and α2 chains by these agents still retained fibroblast chemotactic activity (Table 4).

Table 3. Chemotactic response of different human fibroblast lines of collagen

Fibroblast line	Chemotactic activity, fibroblasts/20 OIF* (mean ± SEM)	
	Buffer	Collagen
D-21	7 ± 1	82 ± 4
S-21	4 ± 1	69 ± 5
S-19	5 ± 1	61 ± 7
S-14	3 ± 1	64 ± 8
S-10	6 ± 2	79 ± 8
Y-51	9 ± 2	65 ± 10

Six different fibroblast lines were tested on the same experimental day to compare responsiveness to 0.9 μM type I lathyritic chick skin collagen.

* Oil immersion fields.

Table 4. Fibroblast chemotactic assay of degraded α chains

Substance tested	Concentration, mg/ml	Chemotactic activity, fibroblasts/20 OIF* (mean ± SEM)
α1 Pepsin	0.92	65 ± 6
α2 Pepsin	0.92	67 ± 7
α1 CNBr	0.67	36 ± 3
α2 CNBr	0.67	44 ± 5
Pepsin control		7 ± 2
CNBr control (α1)		8 ± 2
CNBr control (α2)		6 ± 2
Buffer control		8 ± 1

See *Materials and Methods* for conditions of pepsin and CNBr treatment of α chains from type I lathyritic chick skin collagen.

* Oil immersion fields.

In addition, peptides generated by degradation of type I, II, and III collagens by bacterial collagenase were also chemotactic for fibroblasts (Table 5). Analysis of fractions obtained by gel filtration on Sephadex G-50 of bacterial collagenase-digested type I, II, and III collagens indicated that peptides of various sizes so generated were chemotactic for fibroblasts (Fig. 2).

Noncollagenous peptides generated by pepsin treatment of bovine serum albumin or ovalbumin were not chemotactic for fibroblasts (data omitted).

Tri- and Dipeptides. It was important to determine the amino acid composition and size of peptides fibroblasts would recognize as chemotactic. Several synthetic tri- and dipeptides, many with sequences of amino acids found in collagen, were assayed for fibroblast chemotactic activity. The three peptides containing hydroxyproline, Gly-Hyp, Gly-Pro-Hyp, and Pro-Hyp, were chemotactic at concentrations of 2.5 and 12.5 mM (Table 6). At 25 mM concentration, migration of fibroblasts to these peptides was diminished, perhaps due to rapid diffusion of the peptides across the polycarbonate filters (Table 6). Gly-Phe-Ala, a sequence also found in collagen, was chemotactic at a concentration of 12.5 mM (Table 6). A small amount of fibroblast chemotactic activity was observed with Pro-Gly-Gly at a concentration of 12.5 mM (Table 6). Hydroxyproline and other amino acids common to collagen at concentrations of 100 and 10 mM were not themselves chemotactic for fibroblasts (data not shown).

Table 5. Effect of degradation of human collagens by bacterial collagenase on fibroblast chemotaxis

Substance tested*	Chemotactic activity, fibroblasts/20 OIF† (mean ± SEM)
Type I	51 ± 6
Type I + collagenase	46 ± 5
Type II	57 ± 6
Type II + collagenase	45 ± 3
Type III	49 ± 4
Type III + collagenase	58 ± 5
Buffer	9 ± 1
Buffer + collagenase	7 ± 1

* Each type collagen (900 μg in 1 ml) or buffer was incubated for 18 hr at 37° with bacterial collagenase (18 μg in 0.1 ml). Collagenase-treated samples were heated at 56° for 3 hr to inactivate collagenase.

† Oil immersion fields.

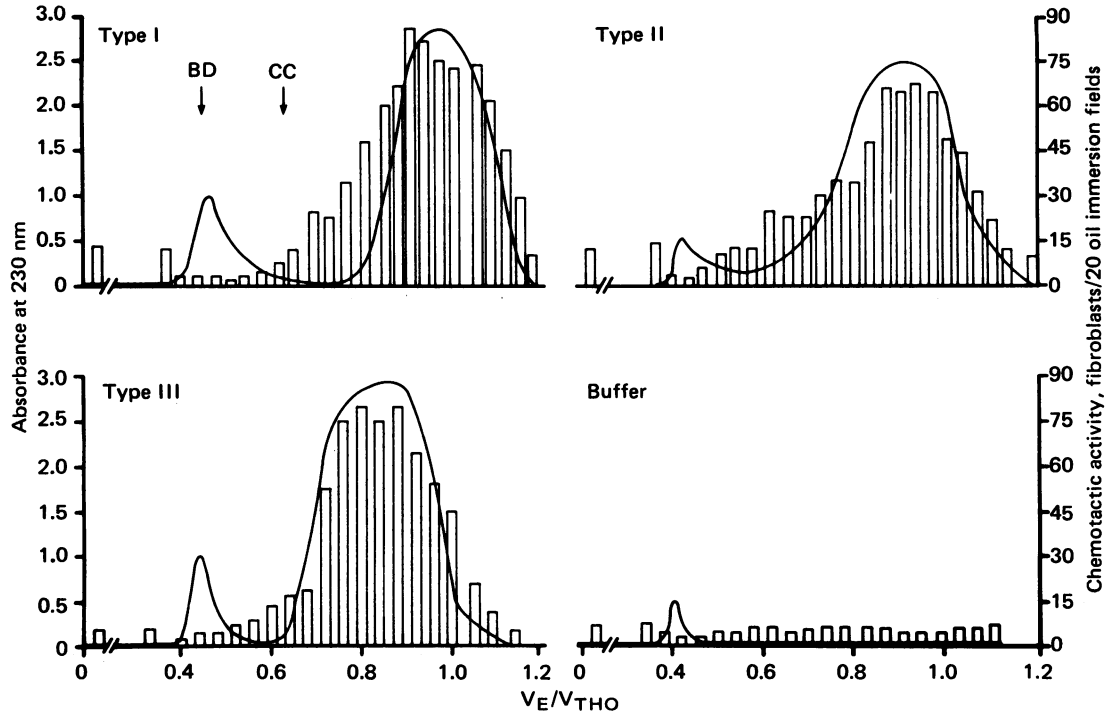

FIG. 2. Human type I and II collagens (*Upper*) and human type III collagen and buffer (*Lower*) were reacted with bacterial collagenase and fractionated on the same Sephadex G-50 column as described in *Materials and Methods*. Column fractions were assayed for fibroblast chemotactic activity. Line, absorbance at 230 nm; bars, chemotactic activity; BD, blue dextran; CC, cytochrome *c*.

DISCUSSION

Human type I, II, and III collagens and isolated α chains from these collagens are chemotactic for human dermal fibroblasts *in vitro*.

The reasons for the difference in chemotactic potency of native collagen and α chains are not apparent from our data. Perhaps the ordered helical configuration of the native collagen molecule in contrast to the random coil structure of α chains more readily exposes the essential amino acid sequence(s) so that it (they) can make contact more efficiently with the appropriate receptor(s) on fibroblast membranes. We have observed a similar difference between the chemotactic potency of native type I collagen and its constituent α chains for human peripheral blood monocytes (22).

Perhaps of an even greater interest is our observation that synthetic tri- and dipeptides containing hydroxyproline, isolated α chains, and smaller peptides derived from degradation of collagen by digestion with CNBr, pepsin, or bacterial collagenase are also chemotactic for fibroblasts. These findings suggest that many regions of the collagen chains contain amino acid sequences capable of inducing fibroblast chemotaxis.

Table 6. Chemotactic response of fibroblasts to di- and tripeptides

Peptide tested*	Chemotactic activity, fibroblasts/20 OIF† (mean ± SEM) 0.25 mM	2.5 mM	12.5 mM	25 mM
Gly-Pro	6 ± 1	4 ± 1	15 ± 2	21 ± 2
Gly-Ile	6 ± 1	4 ± 1	6 ± 1	3 ± 1
Pro-Ile	3 ± 1	3 ± 1	13 ± 2	3 ± 1
Pro-Hyp	7 ± 1	45 ± 5	55 ± 4	46 ± 5
Gly-Hyp	5 ± 1	35 ± 5	70 ± 8	12 ± 1
Gly-Pro-Ala	6 ± 2	3 ± 1	11 ± 2	4 ± 1
Gly-Leu-Tyr	5 ± 1	3 ± 1	3 ± 1	6 ± 1
Gly-Phe-Ala	3 ± 1	9 ± 1	47 ± 3	10 ± 1
Pro-Gly-Gly	7 ± 1	3 ± 1	28 ± 4	12 ± 7
Gly-Pro-Hyp	6 ± 1	20 ± 2	48 ± 3	30 ± 3

* Synthetic di- and tripeptides in the same assay were tested for fibroblast chemotactic activity at the concentration indicated. Fibroblast migration to buffer was 5 ± 1.

† Oil immersion fields.

Several characteristics of the fibroblast membrane receptor involved in the chemotactic response to collagen are apparent from our studies. First, there appears to be a common receptor for type I, II, and III collagens and α1(I); second, hydroxyproline is an important and perhaps essential constituent of the amino acid sequence(s) recognized by the receptor; and third, the receptor involved in the chemotactic response of fibroblasts to collagen is probably different from the receptor involved in the chemotactic response to LDCF-F.

The significance of the observation that collagen and derived peptides are chemotactic for human dermal fibroblasts can only be speculated upon at present. However, the following points might be relevant. In normal connective tissues, collagen fibrils are tightly arranged into larger bundles or fibers. Glycosaminoglycans are closely associated with the packed collagen fibers. The glycosaminoglycans and the structural nature of the fibers may effectively "shield" collagen from fibroblasts so as not to provide a chemotactic stimulus. After tissue damage and during inflammatory reactions, collagen fibers may become "unshielded" by the concerted action of various lysosomal glycosidases and hydrolases and be degraded by specific collagenases present in neutrophils, macrophages, and other cells (23–27). After collagen fibrils are cleaved by collagenase, the digestion products denature under the physiologic conditions of body temperature, pH, and ionic strength (28). These products can be degraded further by the action of nonspecific proteases (28). Our findings *in vitro* suggest that peptides generated by the action of collagenase and nonspecific proteases on collagen could function as chemotactic stimuli to effect fibroblast migration to sites of inflammation *in vivo*.

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