Binding of Chemotactic Collagen-Derived Peptides to Fibroblasts

THE RELATIONSHIP TO FIBROBLAST CHEMOTAXIS

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ABSTRACT We previously showed that collagen, α-chains, and collagen-derived peptide fragments induce chemotactic migration of human fibroblasts in vitro. We now describe biochemical and immunological evidence showing there are binding sites for collagen peptides on fibroblast membranes.

By the use of ^{14}C-labeled $\alpha 1(I)$ chain, binding to intact fibroblasts was demonstrated. The process was reversible, and time- and fibroblast concentration-dependent. Scatchard plot analyses of the data obtained for the binding of $\alpha 1(I)$ suggested that there are $\cong 16 \times 10^6$ binding sites per fibroblast with an association constant of 1.1×10^7/M for $\alpha 1(I)$. Dissociation of the bound radioactivity and subsequent chromatographic analysis on agarose A-1.5 m revealed that the $\alpha 1$ was unaltered. The binding of ^{14}C-labeled $\alpha 1$ was inhibited by each of the CNBr peptides derived from $\alpha 1$ chain of chick skin collagen and CNBr peptide mixtures of various genetic types of collagen chains.

Immunofluorescence studies with anti-$\alpha 1$ antibody showed that $\alpha 1$-treated fibroblasts exhibited strong immunofluorescence. The intensity of fluorescence was markedly diminished by prior absorption of the antibody with $\alpha 1$. The $\alpha 1$-treated cells stained with preimmune sera did not show significant fluorescence.

Dose-response curves of fibroblast chemotaxis induced by $\alpha 1$ and the binding of $\alpha 1$ by fibroblasts correlate closely. Furthermore, the potency of $\alpha 1$-CNBr peptides as chemotactic agents correlates with their ability to inhibit the binding of labeled $\alpha 1(I)$. These data suggest the hypothesis that collagen-derived peptides cause fibroblast chemotactic migration by acting on fibroblast membranes.

INTRODUCTION

Fibroblasts play an important role in the synthesis of new connective tissue elements at the site of tissue injury and inflammation. Several laboratories (1–5) have demonstrated that fibroblasts are capable of migrating in vitro or in vivo but the stimuli which cause migration of fibroblasts have not been thoroughly investigated. Recently, studies in our laboratories demonstrated that collagen and its constituent polypeptide chains, $\alpha 1$ and $\alpha 2$ as well as CNBr peptides derived from the denatured chains serve as chemotactic stimuli for fibroblast migration in vitro (6). The mechanism by which collagen and its degradation products attract fibroblasts and cause migration, however, has not been clarified.

In this study, we investigated the first step in the chemotactic response—the interaction of the chemotactic agent with the responding cell. We have employed ^{14}C-labeled $\alpha 1$ chain of type I collagen to directly label fibroblast receptor sites and to study the interaction of various collagen-derived peptides with the receptors that may serve as recognition sites for the chemotactic response. Our data suggest that fibroblasts possess membrane receptor(s) for collagen and its denatured peptide chains. Immunofluorescence studies with specific anti-$\alpha 1$ antibody also support the hypothesis.

METHODS

Preparation of cultured human fibroblasts. Dermal fibroblasts obtained from punch biopsies of the skin of normal adults were grown in monolayer culture by a standard technique. Cultures were maintained in plastic Petri dishes

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(100 × 20 mm) (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in a humidified atmosphere containing 5% CO_2. Eagle's minimal essential medium supplemented with nonessential amino acids, ascorbic acid (50 μg/ml), $NaHCO_3$, Hepes (Calbiochem, La Jolla, Calif.) buffer (pH 7.2), penicillin (100 U/ml), streptomycin (100 μg/ml), and heat-inactivated fetal calf serum (15%) was used as maintenance medium.

Confluent monolayers of fibroblasts were routinely dispersed by pouring off the maintenance medium, washing the monolayers twice with 10 ml of 0.015 M phosphate/ 0.135 M NaCl, pH 7.4 (PBS),[1] and then detaching them at room temperature with 1 ml of 5 mM EDTA in PBS. The cells, released within 2 min, were collected in 10 ml of maintenance medium, centrifuged at room temperature at 300 *g* for 10 min, and washed once with serum-free maintenance medium and PBS. The clumped cells were removed by low-speed centrifugation (100 *g*) for 2 min. Fibroblasts were then resuspended to a concentration of 10^6 cells/ml in PBS. Trypan blue (1% in PBS) exclusion tests showed >95% viable cells. In all experiments, fibroblasts were obtained from cultures of 10–12 passages.

Preparation of chick skin collagen, α-1 chains, and CNBr peptides. Neutral salt-soluble collagen was extracted from the skin of 3-wk-old White Leghorn chicks which has been rendered lathyritic by administration of β-aminopropionitrile (Aldrich Chemical Co., Inc., Milwaukee, Wis.) for 2 wk. Extracted collagen was purified by repeated precipitations with NaCl from neutral and acid solutions as described (7). Purified α-chains were prepared by chromatography of heat-denatured collagen on carboxymethyl cellulose (7). For preparation of radiolabeled α1, the animals had been injected intraperitoneally with 100 μCi of [^{14}C]glycine (sp act = 49.73 μCi/mmol, New England Nuclear, Boston, Mass.) in 1 ml of sterile 0.9% NaCl once a day for 3 days before they were killed. The specific activity of the ^{14}C-labeled α1 chains was 8 Ci/mol.

The CNBr peptides of the α1 chain were prepared by a combination of ion-exchange and molecular-sieve chromatography of CNBr digests of the α1 chain on various resins as described in detail elsewhere (8). Human collagens and CNBr peptide preparations are described elsewhere (6). All the collagen chains and their peptides were heat denatured immediately before adding to the binding assays.

Binding experiments. Assays for the binding of the ^{14}C-labeled α1 chain to washed fibroblasts were performed in PBS containing 0.5% bovine serum albumin. The assay mixtures consisted of 25 μg of heat-denatured [^{14}C]glycine-labeled α1 in a final volume of 0.2 ml containing 5 mM $CaCl_2$ and various amounts of washed fibroblasts for the appropriate periods of time as indicated. In some experiments, various other CNBr peptides were added to the mixtures. After specified intervals, incubations were terminated by passing the mixture through a 0.65-μm millipore filter which had been soaked in PBS containing 0.5% bovine serum albumin (Millipore Corp., Bedford, Mass.) under reduced pressure. The fibroblasts were washed with 10 ml of PBS containing 0.5% bovine serum albumin (to eliminate the nonspecific binding of α1 chain to the container and filters) and counted in a Nuclear-Chicago scintillation counter (Nuclear Chicago, Des Plains, Ill.) with Aquasol (New England Nuclear) as solvent.

Identification of fibroblast-bound radioactivity. The bound radioactivity was dissociated from fibroblasts by soaking the filters containing the fibroblasts in 20 ml of PBS containing 1.25 mg/ml of unlabeled α1 chain at 37°C for 1 h with constant shaking. The dissociated material was chromatographed on agarose A 1.5m (Bio-Rad Laboratories, Richmond, Calif.) and carboxymethyl cellulose columns. The details of these procedures have been reported (9).

Preparation of antiserum against α1 chain and collagen. Antisera were raised in rabbits immunized with 1 mg α1 chains dissolved in 0.5 ml of PBS and emulsified in an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). The details of this procedure as well as the determination of the antibody titer and specificity have been documented (9).

Immunofluorescence studies. Smears of washed fibroblasts were prepared on glass microscope slides. The dried smears were washed with PBS and fixed with 1% paraformaldehyde for 1 min. The washed, fixed smears were treated with 1% paraformaldehyde for 1 min. The washed, fixed smears were treated with a 1 mg/ml solution of α1 chain or collagen in PBS for 20 min at room temperature in a high humidity chamber. The treated smears were then washed twice with PBS, incubated with several drops of antiserum either to the α1 chain or to collagen for 20 min at room temperature, washed twice with PBS, and finally treated with a 1:10 dilution of fluorescein-conjugated goat anti-rabbit immunoglobulin (Ig) Kalkstad Laboratories, Chaska, Minn.) for 20 min. At the end of incubation, the smears were washed twice with PBS and mounted with glass coverslips using one drop of Gelvatol (Monsanto Co., St. Louis, Mo.). The smears were examined with a fluorescence microscope (Carl Zeiss, New York).

Fibroblast chemotaxis assay. The procedure used was described in detail elsewhere (6). Briefly, fibroblast chemotaxis was assayed using blind-well modified Boyden chemotaxis chambers (Duke University Surgical Instrument Shop, Durham, N. C.) and polycarbonate filters containing 8-μm pores (Wallabs, Inc., San Rafeal, Calif.). Substances being tested for chemotactic activity were dissolved in serum-free maintenance media and placed in the lower compartment of the chambers. The upper compartment contained the fibroblast suspension in maintenance media. Loaded chambers were then incubated at 37°C for 150 min in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, polycarbonate filters were removed, fixed for 15 s in absolute ethanol, and stained with hematoxylin. Chemotactic activity was quantiated by counting nuclei of fibroblasts on the lower surface of the filters in 20 oil immersion fields (×1,000). All samples were assayed in triplicate, and final activity was expressed as the mean±SEM of the replicates.

RESULTS

Binding of ^{14}C-*labeled α1 chain to fibroblasts.* In preliminary experiments, we determined that dispersion of fibroblasts by treatment with trypsin or crude collagenase yielded inconsistent results with regard to the amount of ^{14}C-labeled α1 binding and the chemotactic migration. The amount of binding and cell migration decreased as a function of the duration of the exposure to trypsin. The results obtained by using EDTA-dispersed fibroblasts were consistent, therefore, we elected to employ EDTA-dispersed cells for the present studies.[2]

[1] *Abbreviation used in this paper*: PBS, 0.015 M phosphate/ 0.135 M NaCl, pH 7.4.

[2] Since the completion of these studies, we have found that we could obtain comparable and reproducible results by using 0.25% trypsin, limiting exposure of the cells to trypsin for 3 min, and by allowing "recovery" for 30 min at 37°C.

TABLE I
Binding of ^{14}C-Labeled αl to Human Dermal Fibroblasts

Treatment	^{14}C-labeled αl bound	Counts per minute
	%	
^{14}C-labeled αl, 25 μg	100	386
+αl, 25 μg	101	389
+αl, 125 μg	73	282
+αl, 250 μg	54	208
+αl, 1.25 mg	52	201
+αl, 2.5 mg	31	119

^{14}C-labeled chick skin αl (25 μg, ≅2,000 cpm) was incubated with fibroblasts (5 × 10^5) for 30 min at 37° with gentle shaking. In some experiments nonradioactive αl was added in amounts indicated and incubation continued for an additional 30 min. Fibroblast-bound ^{14}C-labeled αl was isolated by membrane filtration. The experiments were performed in dpublicate tubes. The radioactivity counts are not corrected for nonspecific binding.

Incubation of human dermal fibroblasts with ^{14}C-labeled αl resulted in binding (Table I). Binding was reversible; the bound radioactivity could be displaced with an excess of unlabeled αl (Table I). The dissociation of the bound radioactivity by unlabeled αl occurred rapidly at 37°C (Fig. 1). As shown in Table I, some of the bound radioactivity could not be displaced with a 100-fold excess of unlabeled αl (31%). In the subsequent binding experiments, therefore, duplicate incubations were performed with and without the addition of a 100-fold excess of unlabeled αl, and the amount of labeled αl bound to fibroblasts in the presence of 100-fold excess of unlabeled αl was considered as nonspecific binding. Each value for ^{14}C-labeled αl bound by fibroblasts was corrected by subtracting the value obtained in a duplicate incubation containing excess unlabeled αl.

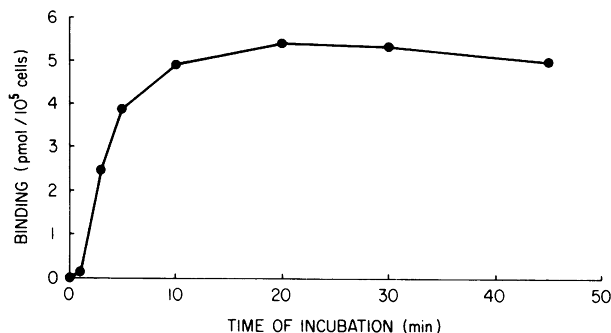

FIGURE 2 Time-dependent binding of ^{14}C-labeled αl by fibroblasts. 25 μg of ^{14}C-labeled αl was incubated with the washed fibroblasts (3 × 10^5) at 37°C. At the time indicated, fibroblasts-bound ^{14}C-labeled αl was isolated by membrane filtration. Each point is the mean of duplicates. All values were corrected for nonspecific binding.

The binding of αl chain to fibroblasts is a time-dependent process. The time needed to reach the maximal binding was 20 min at 37°C (Fig. 2) which is longer than the time required for a maximal binding of αl chain to platelets (9). The binding of αl to fibroblasts is also dependent on the concentration of fibroblasts (Fig. 3). Binding increased with the increase in the number of fibroblasts up to 5 × 10^5 cells and then leveled off in the presence of 25 μg ^{14}C-labeled αl. In most experiments, 1 to 2 × 10^5 fibroblasts were used.

The binding of ^{14}C-labeled αl to fibroblasts is dependent on CA^{++} concentration. Maximal binding was observed in the presence of 5 mM Ca^{++}. Higher concentrations were inhibitory (Fig. 4).

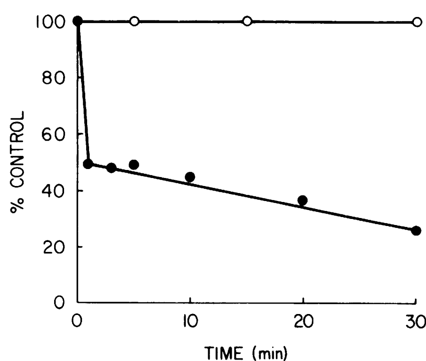

FIGURE 1 Dissociation of fibroblast-bound αl chains. ^{14}C-labeled αl was preincubated with the washed fibroblasts (3 × 10^5) in 0.1 ml of PBS for 20 min at 37°C. At 0 time, 2.5 mg of unlabeled αl dissolved in 0.2 ml of the same buffer was added and incubated for the additional times indicated (●). To the controls was added 0.2 ml of the same buffer only (○). Each point represents separate duplicate incubations.

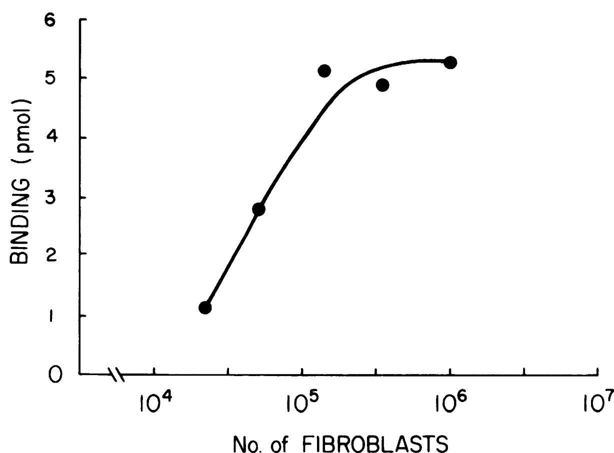

FIGURE 3 Effect of the number of fibroblasts on binding of ^{14}C-labeled αl. Labeled αl (25 μg) was incubated at 37° for 20 min with varying numbers of fibroblasts as indicated. All values were corrected for nonspecific binding.

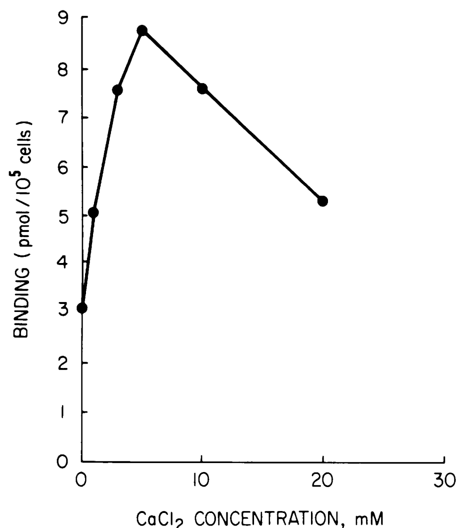

FIGURE 4 The effect of calcium ion on the binding of ^{14}C-labeled α1 to fibroblasts. Washed fibroblasts (3×10^5) were incubated with various concentrations of $CaCl_2$ at 37°C for 20 min. All values were corrected for nonspecific binding.

Determination of association constant. To determine the average number of binding sites per fibroblasts and their affinity as measured by labeled α1(I), the amount of α1 chain bound to fibroblasts was determined as a function of free α1 added to the system. The resulting data were analyzed by the method of Scatchard (10) (Fig. 5). The number of α1 molecules bound to fibroblasts at saturation was calculated from the intercept with the abscissa. From a number of experiments using different fibroblast sources, it was determined that there was a single population of $\cong 16 \times 10^6$ binding site per fibroblast with an association constant of 1.1×10^7/M. The association constant is about 20 times less than that determined for platelets (9).

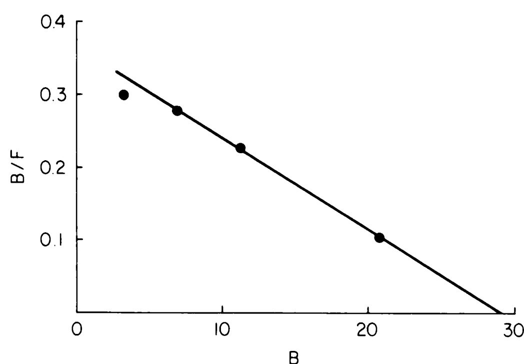

FIGURE 5 Scatchard plot. The ratio, bound:free ^{14}C-labeled α1 is plotted as a function of bound α1 according to the method of Scatchard (10). B, amount of α1 bound (picomoles) to fibroblasts; F, amount of free α1 in nanomoles. The intercept with the abscissa, 29 pmol/1.1×10^6 fibroblasts, corresponds to an approximation of the binding capacity of fibroblast at saturation. From the Avogadro number (6.02×10^{23}), the number of binding sites per fibroblasts was calculated to the $\cong 16 \times 10^6$.

TABLE II

*The Effect of Collagen, α1, α2, β_{12}, Various CNBr Peptides, and Unrelated Proteins on the Binding of ^{14}C-Labeled α1 to Fibroblasts**

Test substances	Inhibition (of specific binding)
	%
Control	0
Lathyritic chick skin collagen	63
α1	84
α1-CB1	18
α1-CB2	17
α1-CB3	25
α1-CB4	20
α1-CB5	18
α1-CB6	22
α1-CB7	50
α1-CB8	43
α2	97
β_{12}	96
Human collagen	—
α1(I)	86
α1(I) CNBr peptides	29
α2	91
α2 CNBr peptides	86
α1(II)	24
α1(II) CNBr peptides	41
α1(III)	89
α1(III) CNBr peptides	80
Hemoglobin	2
Ovalbumin	5
Insulin	5

Washed fibroblasts (5×10^5) were incubated with the test substances as listed in the table for 20 min at 37°C. After the addition of ^{14}C-labeled α1, the mixtures were incubated at 37°C for another 20 min period. The bound α1 was isolated by membrane filtration. Values are expressed as the percent of control experiments. All the substances were tested 100-fold M excess of ^{14}C-labeled α1 except lathyritic chick skin collagen was tested at 50-fold M excess.

Identity of radiolabeled material after binding to fibroblasts. Approximately 70% of the radioactivity bound to fibroblasts was eluted by extracting the fibroblasts with a 100-fold excess of unlabeled α1. The dissociated material was lyophilized. A portion was dissolved in 0.01 M Tris 1 M $CaCl_2$, pH 7.4, applied to a 2×110-cm column of agarose A 1.5m and eluted with the same buffer. Only one radioactive peak which cochromatographed with unlabeled α1 chain was observed (data not shown). The recovery of the radioactivity was $\cong$95%. Chromatography of a separate portion

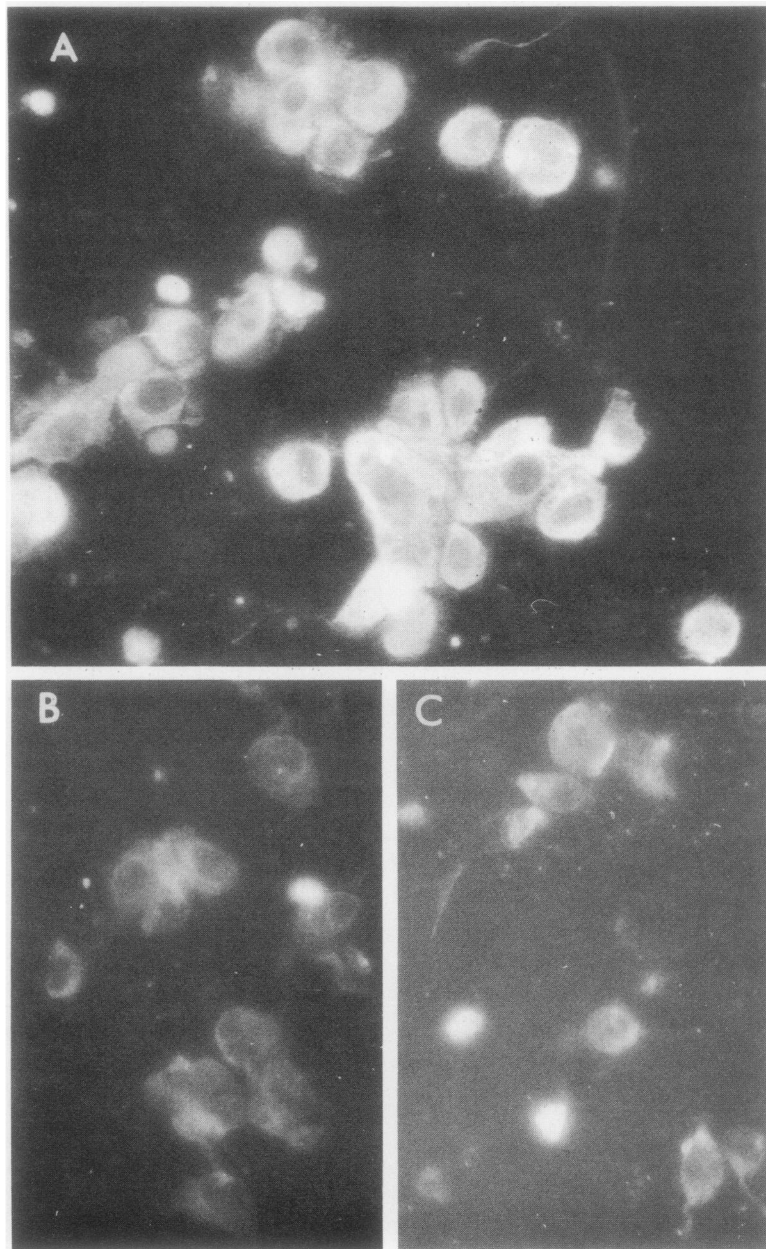

FIGURE 6 Immunofluorescence study of collagen $\alpha 1$ chain binding by fibroblasts. Dermal fibroblasts were treated with $\alpha 1$(I) chain, washed and incubated with antiserum to $\alpha 1$ (A); preimmune rabbit serum (B); and anti-$\alpha 1$ antiserum absorbed with $\alpha 1$ (C); then with fluorescein-conjugated goat antirabbit IgG. Untreated fibroblasts incubated with anti-$\alpha 1$ antiserum showed weak fluorescence similar to that shown in C.

of the displaced material on a column of carboxymethyl cellulose also yielded a single peak of radioactivity coeluting with unlabeled $\alpha 1$ (figure not shown). These results indicate that the $\alpha 1$ chain remained unaltered during the binding reaction.

Inhibition studies. In an attempt to identify the interaction site(s) between $\alpha 1$ and fibroblasts, intact collagen, $\alpha 1$, $\alpha 2$, β_{12}, and various $\alpha 1$ CNBr peptides were tested for their ability to inhibit binding of ^{14}C-labeled $\alpha 1$ to fibroblasts. The results are presented in Table II. When tested with a 100-fold molar excess, all of the collagen-derived peptides were capable of inhibiting the binding to some degree, although larger fragments tended to be more potent as an inhibitor than

smaller fragments. The $\alpha 1$ and $\alpha 2$ chains of type I, α-chains of type II and III human collagen as well as the CNBr peptide mixtures derived from human α-chains produced similar results (Table II). It is of interest that the inhibitory property is not confined to the $\alpha 1$-CB5 glycopeptide, which was shown to be specifically involved in collagen-platelet interactions (9). Several proteins unrelated to collagen had no inhibitory effect on the $\alpha 1$ binding (Table II). These results are consistent with our observation that fibroblast chemotaxis can be induced by the CNBr peptides as well as native types I, II, and III collagens and their α-chains but not by the proteins unrelated to collagen (6).

Immunofluorescence studies. Further evidence that the $\alpha 1$ chain binds to fibroblasts was obtained by immunofluorescence studies. The fibroblasts treated with $\alpha 1$ followed by antiserum to $\alpha 1$ and then fluorescein-conjugated goal antirabbit IgG fluoresced intensely (Fig. 6A), as compared to the absence of fluorescence in $\alpha 1$-treated fibroblasts treated with normal rabbit serum (Fig. 6B) and to the $\alpha 1$-treated fibroblasts stained with anti-$\alpha 1$ antiserum which had been absorbed with $\alpha 1$ (Fig 6C). Untreated fibroblasts incubated with anti-$\alpha 1$ antiserum showed only weak fluorescence (not shown in figure). These results support the concept that $\alpha 1$ binds to the surface of fibroblasts.

Relationship of the $\alpha 1$ binding with chemotaxis. The various concentrations of $\alpha 1$ used to induce fibroblasts chemotaxis and the amounts of $\alpha 1$ bound to intact fibroblasts at each concentration of $\alpha 1$ are depicted in Fig. 7. The dose-response curves for chemotaxis and the binding show a correlation suggesting a relationship between the two phenomena. The concentrations of $\alpha 1$ which induce fibroblast chemotaxis correlate well with those associated with binding. The relationship chemotaxis correlate well with those associated with

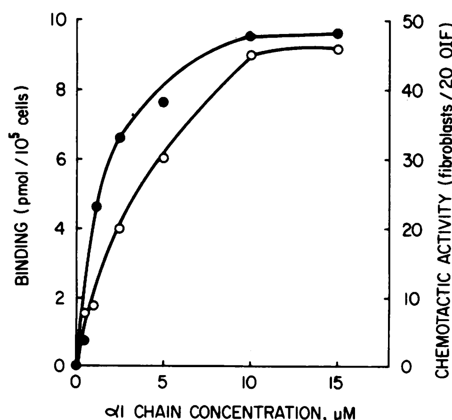

FIGURE 7 Comparison of fibroblasts chemotaxis induced by $\alpha 1$ (O) and the binding of ^{14}C-labeled $\alpha 1$ by fibroblasts (●). OIF, oil immersion field.

TABLE III
Correlation of Fibroblast Chemotaxis and Inhibition of $\alpha 1$ Binding by Collagen Peptides

Test substances	Percentage of inhibition of $\alpha 1$ binding	Chemotactic activity
	%	*Fibroblasts/20 oil immersion field*
Control	0	2±1
$\alpha 1$-CB1,		
30 μM	10	3±1
60 μM	18	14±2
$\alpha 1$-CB3,		
30 μM	10	2±1
60 μM	22	50±6
$\alpha 1$-CB8,		
30 μM	12	12±1
60 μM	38	78±6

binding. The relationship between chemotactic property and inhibition of $\alpha 1$ binding by various CNBr peptides was also investigated (Table III). The largest peptide, $\alpha 1$(I)-CB8, with a mol wt = 24,000 was more effective than the smallest peptide, $\alpha 1$(I)-CB1, with a mol wt = 1,500 as a chemotactic stimulus (Table III) and as an inhibitory agent (Table II). The peptide, $\alpha 1$(I)-CB3, with a mol wt = 13,000 was intermediate in both respects (Tables II and III). These observations suggest the possible biologic significance of these binding sites in inducing fibroblast chemotaxis, as has been suggested for similar correlation between insulin binding and glucose oxidation in the isolated fat cell (11) and between binding of *N*-formylmethionyl peptides and polymorphonuclear leukocyte chemotaxis (12).

DISCUSSION

The results of biochemical and immunological studies demonstrate that binding sites for the $\alpha 1$ chain and collagen-derived peptides of collagen are present on fibroblasts. The binding of $\alpha 1$ chain to fibroblasts is reversible, and the bound ^{14}C-labeled $\alpha 1$ chain can be displaced from fibroblasts by unlabeled $\alpha 1$ chain. Scatchard plot analyses indicate that fibroblasts possess a single population of binding site(s) with an association constant of 1.1×10^7/M for the interaction with $\alpha 1$ chain, and that at saturation an average of 16×10^6 molecules of $\alpha 1$ are bound per fibroblast (10). However, it is still possible that there may be an additional population of binding sites with a much higher affinity as has been reported in other systems (13–15). Because of the limitations in obtaining $\alpha 1$ chain of sufficiently high specific radioactivity by in vivo labeling of collagen, it was not possible to examine binding at much lower concentrations of $\alpha 1$ chain than used in this study.

Inhibition studies using various CNBr peptides in-

dicate that virtually every one of the peptides interferes with the α1 binding. These results are consistent with the observation that the α1-CNBr peptides are active in inducing fibroblast chemotaxis (6). Furthermore, the ability of various CNBr peptides to inhibit α1 binding correlates with their chemotactic property.

A good correlation between the concentrations of α1 inducing fibroblast chemotaxis and those associated with the binding at the cell was obtained (Fig. 7). A similar correlation has been reported for the binding of *N*-formylmethionyl peptides by polymorphonuclear leukocytes and the peptide-induced monocyte chemotaxis (12). These observations strongly support the hypothesis that the collagen α1 chain and collagen-derived peptides act on the surface of fibroblasts to initiate the complex series of events culminating in chemotactic migration of fibroblasts. The mechanisms whereby the surface interaction effects the cell migration are not understood at present but are under investigation in our laboratory.

A comment should be made pertaining to the ability of cells involved in the secretion of collagen to respond chemotactically to exogenous collagen peptides. In normal connective tissue, collagen fibers are formed in close association with mucopolysaccharides which may serve to mask the collagen and prevent interaction with and chemotaxis of fibroblasts. At sites of tissue damage and inflammation the collagen fibers may become unmasked by the action of various lysosomal glycosidases and hydrolases and the collagen itself may be degraded by specific collagenases. Lysosomal enzymes able to digest mucopolysaccharides and proteins have been shown to reside in human neutrophils (16), and specific collagenases that are able to digest collagen fibrils have been demonstrated in neutrophils and in sites of inflammation (17, 18). The cleavage of collagen by a specific collagenase renders the molecules susceptible to further denaturation at physiologic ionic strength, pH, and body temperature and the denatured molecules become susceptible to further degradation by nonspecific proteases (19). Our results obtained in this study suggest the possibility that such degradation products of collagen in inflammed tissues may serve as chemotactic stimuli for tissue fibroblasts.

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