Interaction between fibrinogen and cultured endothelial cells. Induction of migration and specific binding.

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Research Article

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Interaction Between Fibrinogen and Cultured Endothelial Cells

Induction of Migration and Specific Binding

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Abstract

It has been suggested that fibrinogen (fg) or its physiological derivatives influence the motility and growth of endothelial cells (ECs), but direct support for this concept is still lacking. In the present study, the capacity of fg to interact with ECs and induce the migration of ECs was examined.

The capacity of fg to induce EC migration was studied by means of a modification of the Boyden chamber technique. fg in the lower compartment of the chamber caused a time- and concentration-dependent migration of ECs across filters. fg present in equal concentrations above and below the filter increased EC migration, but the maximal effect invariably occurred in the presence of a gradient between the lower and the upper compartments.

Trypsin or plasmin digestion of fg and preincubation of fg with Fab fragments from specific antibody completely abolished fg-induced EC migration.

Dialysis of fg to eliminate small peptides that might contaminate the preparation did not modify fg-induced migration. Plasma obtained from healthy donors induced EC migration, but plasma from an afibrinogenemic patient was completely ineffective. The addition of purified fg to afibrinogenemic plasma restored plasma-induced EC migration. Plasmin degradation fragments D and E, of 100,000 and 50,000 mol wt, respectively, did not induce EC migration. However, fragment E caused dose-related inhibition of fg-induced EC migration.

Direct interaction of highly purified radioiodinated human fg with cultured human and bovine ECs was observed. The binding was time dependent and plateaued at 10 min. Nonlabeled fg in a large molar excess inhibited the interaction, but unrelated proteins, including fibronectin, ovalbumin, and myoglobin, did not. Monospecific Fab fragments directed to fg inhibited binding by 38% at a 50 to 1 molar ratio whereas nonimmune Fab caused only 2% inhibition at a similar concentration. The binding of ^{125}I-fg with ECs was saturable, and an apparent dissociation constant of 0.23×10^{-6} M was estimated from binding isotherms. After 30 min of incubation the interaction between ^{125}I-fg and the cells was completely reversible and displaceable by a large molar excess of unlabeled fg. Autoradiography of the display of EC-bound ^{125}I on polyacrylamide gel showed the constitutive Bβ- and γ-chains of the fg molecule, with a partial loss of the Aα-chain.

Purified fragment E and D were tested for their capacity to inhibit fg binding. At a 1 to 400 ^{125}I-fg-to-fragment molar ratio, fragment E, which also inhibited migration, competed for binding by 44%, but fragment D was completely ineffective.

These data show that fg may specifically associate with ECs and induce migration of these cells; it also appears that the structural requirement of this activity is located in the N-terminal part of the molecule.

Introduction

Factors that contribute to the motility and growth of endothelial cells (ECs)[1] are important mediators for vessel wall repair at sites of injury and neovascularization of tumors.

Fibrinogen (fg) or its derivative fibrin have been recognized as one of these factors for EC activity. Fibrin influences EC migration (1), and exposure of confluent sheets of vascular endothelium to fibrin causes cell contraction and disruption of the cell monolayer (2).

Tumor growth is frequently associated with activation of the clotting system (3), and migration and proliferation of ECs into fibrin surrounding tumor cells could play a role in the neoformation of tumor microvasculature (4). Thus, the fg molecule may express interfaces with ECs and may influence EC migration, but direct evidence supporting this concept is still lacking. An attractive hypothesis is that the participation of the molecule in endothelial function is mediated via specific binding sites; the identification of receptor systems for fg on platelets (5, 6), macrophages (7, 8), and fibroblasts (9, 10) may support this concept.

In the present study we show that native fg induces EC migration and associates specifically with ECs, and that the structural requirement for this activity is located in the E domain of the molecule.

Methods

Materials. The carrier-free ^{125}I (17 Ci/mg) was purchased from Amersham International (Buckinghamshire, England).

Other materials were obtained from the following sources: bovine serum albumin (BSA), trypsin, soybean trypsin inhibitor, and papain from Sigma Chemical Co. (St. Louis, MO); hirudin from Laboratoire Stago (Paris); aprotinin (Inoprol) from Laboratoire Choay (Paris); chloramine T from Eastman Kodak Co. (Rochester, NY); ovalbumin

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1. *Abbreviations used in this paper:* EC, endothelial cell; fg, fibrinogen; MEM, Eagle's minimum essential medium.

from Worthington Biochemical Corp. (Freehold, NJ); and myoglobin and 2-mercaptoethanol from J. T. Baker Chemical Co. (Phillipsburg, NJ). All culture reagents were purchased from Gibco Laboratories (Paisley, Scotland). The plastic flasks and petri dishes came from Falcon Labware, Div. of Becton-Dickinson Co. (Oxnard, CA); plasmin from Kabi AB (Stockholm); and casein from Merck Sharp & Dohme AG (Darmstadt, West Germany). Chemotactic complement components (activated serum) were obtained by incubating normal human serum with zymosan (11); the lymphocyte-derived chemotactic factor(s) was obtained by culturing human lymphocytes with phytohemagglutinin for 3 d (12). Culture supernatants from the human 8387 sarcoma line were used as a source of tumor-derived chemotactic factor(s) (12); fibronectin was purified from fresh human citrated serum by affinity chromatography on gelatin Sepharose (13). Purified von Willebrand factor was a kind gift from Dr. D. Meyer, Hôpital Bicêtre, Le Kremlin Bicêtre (Paris) (14). Heparin was from Crinos S.p.A. Villa Guardia (Como, Italy). Polycarbonate filters for the migration assay were from Nucleopore Corp. (Pleasanton, CA); Diff-Quick was from Harleco, Hartman-Leddon (Gibbstown, NY).

fg and fg derivatives. Human fg was purified according to described procedures (15). Polyacrylamide gel electrophoretic analysis of the purified material in the presence of sodium dodecyl sulfate (SDS) and reducing agents demonstrated only intact, Aα-, Bβ-, and γ-chains. Plasminogen, Factors II, XIII, and VIII, von Willebrand factor, and fibrin monomer could not be demonstrated in any preparation by established criteria. Contaminating fibronectin did not exceed 1% and was eliminated by affinity chromatography on gelatin-Sepharose. fg was trace labeled with carrier-free ^{125}I by the modified chloramine T method incorporating an average of 0.25 mol of iodine/mol of fg (5). The ^{125}I-protein was >91% precipitable in 20% trichloracetic acid. Autoradiography of the ^{125}I-fg after vertical slab polyacrylamide gel electrophoresis in SDS and 2-mercaptoethanol demonstrated that the constituent chains of the labeled fg had the same mobilities as the unlabeled protein. The concentrations of labeled and unlabeled fg were determined spectrophotometrically with an extinction coefficient $E^{0.1\%}_{280\ nm}$ of 1.51.

High molecular weight fragment D 100 (100,000 mol wt) and fragment E 50 (50,000 mol wt) were purified from plasmin digests of native fg obtained in the presence of calcium ions. The purification procedure and the characterization of the fragments have already been described in detail (16).

Preparation of Fab fragments. Antisera to fg were raised in rabbits and adsorbed with plasma from an afibrinogenemic patient to ensure specificity. The IgG fractions were isolated from the antisera and hydrolyzed with papain. The Fab fragments were immunochemically purified by affinity chromatography on fg-Sepharose. The specificity and affinity of these Fab fragments has already been detailed. Typically the preparation contains Fab's directed to major structural domains of the native molecule, namely D and E (16). Sephadex-G-150 chromatography and SDS-polyacrylamide gel electrophoresis verified the absence of nonhydrolyzed IgG and Fc fragments in the Fab preparations. Nonimmune Fab fragments were isolated by carboxymethyl-cellulose chromatography of papain digest of rabbit IgG.

Cells. ECs from human umbilical cords or bovine aortas were obtained and cultured as described previously (17). The cells were grown to confluence on plastic flasks or petri dishes in Eagle's minimum essential medium (MEM) supplemented with 20% fetal calf serum for human or 10% adult bovine serum for bovine ECs.

The continuous cell line EUE was derived from normal human embryo epithelium (18) and cultured in MEM supplemented with 10% heat-inactivated fetal calf serum. The cells were fed twice a week. The experiments reported here were carried out with human ECs in primary culture and bovine ECs in the sixth or seventh passage at confluence. The EUE cells were used at the 20th passage. Ficoll-Hypaque-separated mononuclear cells from normal donors were used as a source of monocytes. Polymorphonuclear leukocytes (PMNs) were obtained by Ficoll-Hypaque separation and sedimentation through dextran for 20 min. Lymphocytes were obtained by adherence on plastic followed by passage through nylon wool columns; monocyte contamination did not exceed 1–2% as assessed by morphology and staining for nonspecific esterase. The procedures used to separate the leukocyte populations are described elsewhere (12, 19, 20).

Chemotaxis assay. EC migration was measured in a blind well Boyden chemotaxis chamber with polycarbonate filters containing 5-μm pores as described previously for monocytes (12) with minor modifications (21). The cells grown to confluence in 25-cm^2 plastic flasks (~2 × 10^6 cells per flask) were washed twice with 5 ml of phosphate-buffered saline (PBS) and detached by brief exposure (4–6 min) to a 1.5 U/ml trypsin and 0.02% EDTA solution. Soybean trypsin inhibitor (0.5 mg/ml final concentration) was added to the cell suspensions to neutralize the trypsin and the cells were immediately centrifuged at 1,200 *g* for 10 min. The cells were resuspended in 0.2 ml of RPMI 1640 medium with 10% fetal bovine serum and seeded in the upper compartment of the chamber. Substances being tested for chemotactic activity were dissolved in 0.2 ml of RPMI 1640 medium with 10% fetal calf serum and placed into the lower compartment of the chambers. In a few assays serum-free medium was used with or without 0.2% BSA. Loaded chambers were then incubated at 37°C for various periods in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, polycarbonate filters were removed, fixed, and stained with Diff-Quick. Chemotactic activity was quantified by a count of ECs on the lower surface of the filters in 20 oil-immersion fields (× 1,000). All samples were assayed in triplicate after they were coded, and final activity was expressed as the mean±SD of the replicates.

To determine the stability of the fg gradient a trace amount of ^{125}I-fg was added (4.2 × 10^6 cpm/200 μl) to unlabeled fg at a concentration of 3 μM in the lower wells. At certain times the radioactivity was determined in the upper well. Migration of monocytes and PMNs was assayed the same way with an incubation time of 90 min (12). Polycarbonate filters used for ECs, monocytes, and PMNs are not suitable for lymphocyte migration since these cells do not remain adherent on the filter surface. Hence lymphocyte migration was evaluated on nitrocellulose filters and quantitated by measurement of the distance into the pores reached by the leading front and by a count of the number of migrated cells, as described elsewhere (12).

Binding of ^{125}I-fg to cells in monolayers. The binding of fg was measured on confluent cells grown on 9.6-mm^2 petri dishes (~5 × 10^5 cells per dish) in an assay medium consisting of PBS supplemented with 1 mM Ca^{2+}, 1 mM Mg^{2+}, and 5% BSA to reduce nonspecific interaction.

The assays were carried out at a constant pH of 7.5 and at 37°C. The wells containing the cells were incubated overnight with serum-free MEM containing 5% BSA before the binding experiments. Under these conditions, the background of nonspecific binding of ^{125}I-fg to the petri dishes was <0.1% of the total radioactivity added.

Radiolabeled fg was diluted with PBS-5% BSA at various concentrations and 1 ml of the solution was layered on the cells. After incubation, binding medium was removed and the cells were gently washed four times with 1 ml PBS 1% BSA. The entire washing phase lasted 30–45 s and did not affect the cell number. After washing was done, the cells were dissolved in either 0.1 M NaOH or 5% SDS containing 2% 2-mercaptoethanol for 10 min at 37°C and counted for radioactivity in a counter (Packard Instrument Co., Inc., United Technologies, Downers Grove, IL). With both solutions the recovery of the radioactive material was complete after extraction (<0.1% of the radioactivity eluted remained on the plate.). Therefore, the extraction solution containing SDS and 2-mercaptoethanol was used for subsequent electrophoresis of the extract at normal pH. Nonspecific binding was determined in the presence of a 100-fold excess of cold fg at each test point in separate assays.

Binding of ^{125}I-fg to cells in suspension. The binding of fg was measured on bovine and human ECs in single cell suspensions. EC suspensions were prepared as described in the previous paragraph for

the chemotaxis assay. The cell pellets were resuspended in PBS supplemented with 1 mM Ca^{2+}, 1 mM Mg^{2+}, and 1% BSA. Cell number and viability were determined by trypan blue exclusion. The binding assays were made by means of a centrifugation technique to separate the free from the bound ligand (5). The cells in suspension (5 × 10^5 cells/ml) were incubated with ^{125}I-fg at constant pH at 37°C. At selected times 50 μl of the cell suspension was layered on 250 μl of 25% sucrose in PBS-1% BSA in conical propylene tubes. After centrifugation for 1 min at room temperature in a microfuge (Beckman Instruments, Inc., Fullerton, CA) the tips of the tubes were cut off with a razor blade and the ^{125}I-fg associated with the cell pellet was determined.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis in Tris/glycine buffer at pH 8.6 was done by means of a vertical slab gel apparatus. Gels and buffer systems were prepared according to the method of Laemmli (15). The upper stacking gel was 3% acrylamide and the lower separating gel was 10% acrylamide. The reduced radioactive material was subjected to electrophoresis and the ^{125}I-display was analyzed by autoradiography.

Results

Fg-induced migration of ECs. We studied the effect of fg on EC migration in a Boyden chamber. Because of the large number of cells required for the migration assay, most of the experiments reported in this study were made with bovine ECs, which are easier to obtain in large numbers than are human ECs. All the experiments reported were repeated at least once, with human ECs giving comparable results.

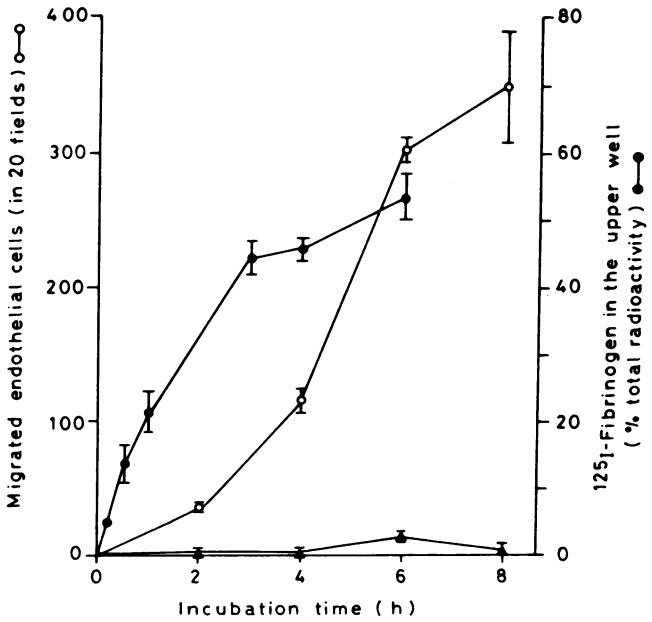

Figure 1. Time course of fg-induced migration of bovine ECs. ECs in suspension (4 × 10^5 in 0.2 ml) were seeded in the upper compartments of the assay chambers. Unlabeled fg (3 μM) was added in the lower compartment with a trace amount of ^{125}I-fg (4.2 × 10^6 cpm/200 μl). At specific times portions of the solution (5 μl) were taken from the upper wells and the radioactivity was determined. Results are expressed as percentage of the total radioactivity added in the lower well (●). Cell migration from the upper to the lower compartments was measured as described in Methods. ○, cells migrated in response to fg. ▲, cells migrated in absence of fg in the lower compartment. Data points are the means of triplicate observations and the bars indicate SD.

In a preliminary series of assays labeled fg was added to the lower compartment of the Boyden chamber and the amount diffused in the upper compartment was measured at different times (Fig. 1). Binding of fg to the filter was very low (~0.2–0.3% of the total added). As shown in fig. 1 concentrations of fg above and below the filter reached equilibrium in ~6 h. Fig. 1 also shows the time course of cell migration in the presence of 3 μM fg. The number of migrated ECs increased with time, reaching a plateau after 6 h of incubation.

In all the experiments performed over more than 1 yr migration was always observed though there was some variability among experiments. The percentage of cells migrating ranged from 3.5 to 7.5% of the total number added. Consistent results were obtained with cells subcultured from the same aorta. In contrast, interindividual variability was apparent when cells from different aortas or from different umbilical cords were compared. There was no apparent consistent correlation with cell passage number. The five different fg preparations we used during this study showed very similar activity.

The number of migrated cells was a linear function of the number seeded from 1–4 × 10^5 (data not shown). Thus, conditions routinely used for assays included 4 × 10^5 cells with a 6-h incubation. Assays were performed routinely with medium supplemented with 10% fetal bovine serum, but comparable results were obtained in a few experiments in which serum-free medium alone or with 0.2% BSA was used. For instance, in a typical experiment 283±21 (mean±SE) ECs migrated in 20 fields to 3 μM fg at 6 h when medium supplemented with 10% fetal bovine serum was used, 274±27 ECs migrated in serum-free medium with added 0.2% BSA, and 203±50 ECs migrated in serum-free medium without additions.

Chemotactic and chemokinetic activity of fg. As reported in Table I, a series of checkerboard experiments was performed to establish whether fg-induced migration depended on a concentration gradient of the protein (chemotactic activity) or was simply due to an increase of cell motility through the filter (chemokinetic activity). Increasing the concentration of fg in the lower compartment resulted in a dose-dependent increase in the number of ECs moving through the filter. fg at equal concentrations above and below the filters appreciably

Table I. Checkerboard Analysis of the Effect of fg on EC Migration

fg in lower chamber	fg in upper chamber (μM)				
	0	0.1	0.3	1	3
μM					
0	14±2	18±5	10±2	4±1	0
0.1	39±17	16±0.5	NT	11±3	7±1
0.3	294±33	NT	153±51	95±6	29±6
1	468±35	384±16	314±12	182±19	35±4
3	495±11	478±13	366±25	345±15	137±39

NT, not tested. Bovine ECs (4 × 10^5 in 0.2 ml) were seeded in the upper compartment of the chamber and after 6 h migration was quantified as described in Methods. Various amounts of fg were added to both upper and lower wells to produce a series of concentration gradients across the membranes. Results are means±SD of triplicate observations. The values in the diagonal, where there is no gradient, show the chemokinetic effect of fibrinogen.

increased migration, as shown by the values in the diagonal. However, maximal migration invariably occurred in the presence of the gradient between the lower and the upper compartment. Thus, although the use of polycarbonate filters does not allow an analysis of data based on leading distance calculations as described by Zigmond and Hirsh (22), the finding of maximal migratory response in the presence of positive gradient suggests a chemotactic response of ECs to fg. These results, therefore, suggest that fg induced EC migration with both chemotactic and chemokinetic activity.

Comparison of fg with other chemoattractants. Fibrinogen was compared with other agents known to be chemoattractants for leukocytes. For migration of monocytes and PMNs the polycarbonate filters were the same as those used for ECs, whereas migration of lymphocytes (nonadherent cells) was assessed in nitrocellulose filters by the leading front method (12). As shown in Table II, none of the stimuli tested had any activity on ECs, but they were significantly active on at least one type of leukocyte (according to their cell specificity). On the other hand, fg had only limited effects on the migration of leukocytes.

Specificity of fg-induced migration. To investigate whether the induction of migration by fg was in fact attributable to this molecule and not to possible contaminants, we performed a series of experiments, using different approaches. As shown in Table III, when fg was dialyzed, the same chemotactic effect was observed, thus excluding the contribution of contaminating small active peptides. Overnight trypsin or plasmin digestion of fg completely abolished this effect. Pre-incubation of fg with Fab fragments at a 10:1 molar ratio completely inhibited chemotaxis. A very small number of ECs migrated in response to fibronectin or von Willebrand factor used under the same conditions. We also tried fibronectin at different concentrations from 5 to 300 μg/ml, and it showed virtually no effect in our experimental conditions.

All these observations were consistent with a specific role for fg in EC migration. The results of experiments in which the plasma of a patient with congenital afibrinogenemia was used supported this concept (Table IV). The level of this plasma was <1 μg/ml as determined by radioimmunoassay (23). When 50% of the medium in the lower chamber was replaced with plasma from healthy donors the number of migrated ECs was not statistically different from the number of cells that migrated in response to purified fg. In contrast, when plasma from the afibrinogenemic patient was used, migration did not occur. When fg was added to the afibrinogenemic plasma at a concentration of 3 μM the induction of migration was partially restored.

Effect of purified fragments E and D. As shown in Table V, fragments D and E were completely ineffective as chemoattractants at concentrations up to 20 and 40 μM, respectively. We tested the effect of fragments E and D on fibrinogen-induced EC migration. As reported in Table V, fragment D was ineffective, but fragment E caused a marked dose-dependent inhibition of fg-induced cell migration.

fg binding to ECs. To assess whether fg-induced EC migration was related to the specific association of the molecule

Table II. Effect of Leukocyte Chemotactic Stimuli and of fg on EC and Leukocyte Migration

Stimulus	ECs	Monocytes	PMNs	Lymphocytes	
				No.	Distance
					μm
Medium	1±0	159±39	25±2	272±13*	102±1
fg	316±14*	309±44*	66±9*	383±35*	117±4*
Activated serum	10±4	874±15*	207±5*	319±23*	127±1*
LDCF	4±3	934±60*	17±2	NT	NT
TDCF	1±1	684±16*	18±3	NT	NT
Casein	7±3	NT	NT	506±17	126±4*

NT, not tested. Bovine ECs (4×10^5 in 0.2 ml) were seeded in the upper compartment of the chamber and after 6 h migration was quantified. Leukocytes were obtained as described in the Methods and seeded (4×10^5 cells in 0.2 ml) in the upper well; migration was assessed as described. Fg was used at 3 μM concentration. Results are means±SD of triplicate observations.
* $P < 0.01$ vs. medium, Dunnett's test.

Table III. Specificity of fg-induced Migration of ECs

Stimulus	ECs
Medium	2±1
fg	142±3
Dialyzed fg	132±6
Trypsin-digested fg	0
Plasmin-digested fg	3±2
Fab antifibrinogen	1±2
Fibronectin	1±0.6
von Willebrand factor	16±1

Bovine ECs (4×10^5 in 0.2 ml) were seeded in the upper compartment and after 6 h migration was quantified. fg was used at 3 μM concentration. Dialzyed fg was obtained by four dialysis periods of 5 h and one overnight against PBS at 4°C in Visking dialysis tubings (Serva, Feinbiochemica, Heidelberg, Federal Republic of Germany) with an exclusion limit of 8,000–15,000 D and was used at a concentration of 3 μM. Trypsin- and plasmin-digested fg was obtained by overnight treatment of fg (3 μM) with 1 mg/ml trypsin and 4 U/ml plasmin, respectively, and subsequent neutralization of the enzymes with 1 mg/ml soybean inhibitor and 100 U/ml aprotinin, respectively. Fab antifibrinogen was incubated with fibrinogen (3 μM) overnight at 4°C at a molar ratio of 10:1. Purified fibronectin and von Willebrand factor were used at concentrations of 0.3 mg/ml and 5 μg/ml (1 U/ml), respectively. Values are means±SD, of triplicates.

Table IV. Effect of Normal Plasma and Plasma Derived from an Afibrinogenemic Patient on EC Migration

Stimulus	ECs
Medium	2±1
fg	69±2
Control plasma	45±5
Afibrinogenemic plasma	1±1
Afibrinogenemic plasma added with fg	26±8

fg was used at a concentration of 3 μM in presence of 5 U/ml heparin. Plasma from a healthy donor and from an afibrinogenemic patient was obtained on heparin (5 U/ml) and used diluted 1:2. Fibrinogen was added to the afibrinogenemic plasma to reach 3 μM concentration. The values are means±SD of triplicate observations from the same experiment. Similar results have been obtained from three other experiments.

Table V. Effect of fg and fg Degradation Fragments E and D on the Migration Response of ECs

fg	Fragment E	Fragment D	ECs
μM	μM	μM	
—	—	—	1±0.7
—	—	20	1±1
—	40	—	0
3	—	—	356±17
3	—	20	349±10
3	40	—	178±28*
3	20	—	190±13*
3	10	—	266±35*
3	2	—	357±5

Bovine ECs (4×10^5 in 0.2 ml) were seeded in the upper compartment of the chamber, and after 6 h, migration was evaluated. Values are means±SD of triplicates.
* $P < 0.01$ vs. fg alone, Dunnett's test.

with the cells, we studied the direct effect of ^{125}I-fibrinogen to ECs.

In all the experiments reported here bovine and human ECs gave similar results. Unless otherwise specified we report the results with human cells.

The direct interaction of ^{125}I-fg with EC monolayers was determined 5 d after plating. After selected incubations at 37°C the cells were rapidly washed and the EC-associated radioactivity was extracted and counted. A typical experiment is shown in Fig. 2, which illustrates the time course of binding in the presence of 0.01 μM ^{125}I-fg. The nonspecific background of interaction was determined in the presence of a 100-fold molar excess of unlabeled fg. Total binding represented between 1 and 2% of the total radioactivity applied, and nonspecific binding corresponded to ~50% of this value. Association of ^{125}I-fg with EC was time dependent and reached saturation after 20 min of incubation, whereas nonspecific binding reached saturation immediately. Specific binding was obtained from

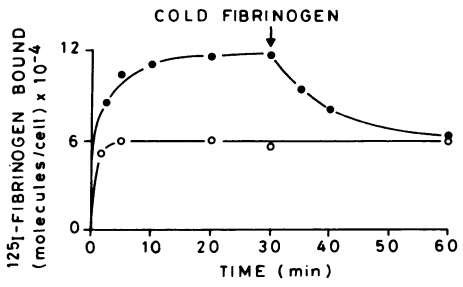

Figure 2. Time course and reversibility of the association ^{125}I-fg with human ECs. Confluent cell monolayers of an average density of 70,000 cells/cm^2 were incubated at 37°C with 0.01 μM ^{125}I-fg in PBS-5% BSA for 30 min. The number of molecules bound per cell was measured in the presence (○) or in the absence (●) of 1 μM unlabeled fg. After 30 min the medium was removed and replaced with buffer containing 1 μM unlabeled fg. At the indicated time, the buffer was removed and the cells were dissolved. Values are means of four replicates; SD ranged from 8 to 10% of the mean. The figure illustrates a typical experiment on one cell culture. Three other experiments gave similar results.

the difference between the two curves and saturated at 55,000±5,000 molecules/cell.

Reversibility of this interaction was assessed by removal of the labeled fg from the monolayer and displacement of the EC-bound ^{125}I-fg by nonlabeled fg at a 100-fold molar excess (Fig. 2). The dissociation of ^{125}I-fg was time dependent and reached the background of nonspecific binding 30 min after addition of the nonlabeled fg. When the interaction with cells in suspension was analyzed, the kinetics of binding was identical (data not shown), but the level of nonspecific binding was only 10 to 15% of total binding.

Some experiments have incubated EC monolayers with 0.01 μM fg at 4°C. The binding was measured at 2, 5, 10, 20, and 30 min. At this temperature the total binding reached saturation after 5 min. At saturation the total binding at 4°C was reduced to ~70% of the total binding obtained in parallel experiments at 37°C (119,308±10,300 molecules/cell at 4°C vs. 164,923±15,230 molecules/cell at 37°C, means±SE of four replicates) while specific binding was not modified (60,692±7,030 vs. 67,077±8,920 molecules/cell).

Specificity of the interaction. Specificity of the binding for fg was established from different experiments. Table VI illustrates the results obtained from a typical experiment, in which unlabeled fg inhibited binding by 38% when present in a large excess, but unrelated proteins such as myoglobin, ovalbumin, and fibronectin had no effect. Fab fragments isolated from monospecific IgG antifibrinogen caused 38% inhibition at a 20:1 molar ratio, a value similar to that obtained in the presence of a large excess of cold fg and which represents specific displaceable interaction. At a similar concentration nonimmune Fab had no effect. When fragments D and E from plasmin digestion of fg were tested for their capacity to inhibit binding, fragment E caused inhibition similar to that of nonlabeled fg, but fragment D had no effect. This was reproducible in five separate experiments, although the level

Table VI. Specificity of the Binding of ^{125}I-fg to Human ECs

Competitor	Final concentration	^{125}I-fg bound	Inhibition
	μM	molecules/cell	%
None		107,800±17,000	—
Unlabeled fg	4	67,300±5,000*	38
Fragment D (Mw 100,000)	16	116,900±15,100	—
Fragment E (Mw 50,000)	16	60,400±3,200*	44
Fab' antifibrinogen	0.8	67,100±8,500*	38
Nonimmune Fab'	0.8	105,700±11,000	2
Myoglobin	10	122,100±10,700	—
Ovalbumin	10	96,700±8,300	10
Fibronectin	1	108,000±12,000	—

The binding of ^{125}I-fg (0.04 μM) was determined on human ECs in monolayers for 30 min at 37°C. Percentage inhibition was calculated relative to the control containing ^{125}I-fg added to the cells in the absence of competitor. Values are means±SEM of quadruplicate determinations.
* $P < 0.01$, Dunnett's test vs. values obtained in the absence of any competitor.

of nondisplaceable bound material differed from one experiment to another. To assess the cell specificity, human and bovine ECs were compared with human embryo epithelial cell (EUE) monolayers. Specific binding of ^{125}I-fg (0.02 μM) was measured for 30 min at 37°C, and EUE cell monolayers were used at confluence at a cell density similar to that of human and bovine ECs. ^{125}I-fg interacted with only ECs of human and bovine origin (40,000±6,000 and 31,000±6,000 molecules/cell, respectively), whereas EUE cells associated very poorly with the molecule (2,000±100 molecules/cell).

Finally, to establish that the bound radioactivity was related to fg, the EC extract was subjected to electrophoresis on SDS-polyacrylamide gel after reduction, and display of ^{125}I was analyzed by autoradiography (Fig. 3). The radiolabeled fg gave the three electrophoretic bands characteristic of the Aα-, Bβ-, and γ-chains (Fig. 3, lane 1). The reaction at the dye front was attributed to the presence of free ^{125}I or small ^{125}I-peptides. The EC extract produced bands in the positions of the Bβ- and γ-chains. The relative integrity of these bands was reduced and the reaction at the dye front increased, suggesting proteolytic degradation (Fig. 3, lane 2). At the same time a partial loss of the Aα-chain was noted. ^{125}I-fg present in the supernatant at the end of the binding experiment was extracted and examined. The three chains were present (Fig. 3, lane 4), and migration of the Aα-chain was affected only by the large amount of BSA present in the binding assay buffer. ^{125}I-labeled fg was removed from the monolayers at the end of the binding assay and a large excess of unlabeled fg was added to the cells in order to displace the ^{125}I-fg specifically bound. The analysis of the displaced ^{125}I-fg present in the supernatant shows that a partial loss of the α-chain was still present, whereas the low molecular weight forms present at the dye-front were completely absent. This indicates that the radioactive material that interacted with EC was fg related and that when the molecule comes into close contact with the cells it is somehow processed. The exact

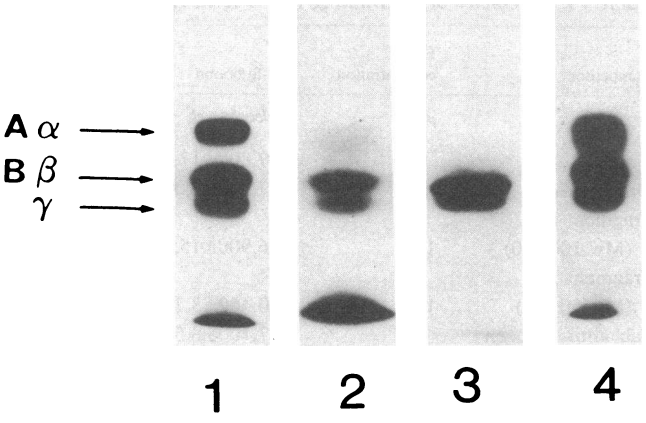

Figure 3. Polyacrylamide gel electrophoresis of ^{125}I-fg. Confluent cell monolayers were incubated at 37°C with 0.3 μM ^{125}I-fg in PBS-5% BSA for 30 min. The medium was then removed and replaced with buffer containing 30 μM unlabeled fg. Samples of the starting ^{125}I-fg, of the cell extract 30 min after binding, and of the supernatant at the end of the binding experiment and 30 min after addition of unlabeled fibrinogen were analyzed on 10% polyacrylamide gel under reducing conditions. Lane 1, starting ^{125}I-fg; lane 2, extract of EC-bound ^{125}I-fg; lane 3, ^{125}I-fg present in the supernatant after displacement with unlabeled fibrinogen; and lane 4, ^{125}I-fg present in the supernatant at the end of the binding experiment.

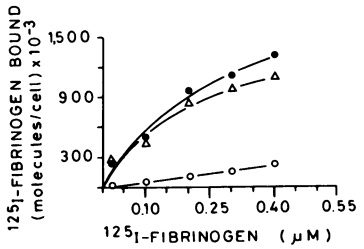

Figure 4. Binding of ^{125}I-fg to human ECs in suspension as a function of ^{125}I-fg concentration. ECs (5 × 10^5 cell/ml) were incubated for 30 min at 37°C with different concentrations of ^{125}I-fg. The number of molecules bound per cell was measured in the presence (○) or absence (●) of a 100-fold molar excess of unlabeled fg. Specific binding (△) was obtained by subtraction of the values obtained in the presence of excess unlabeled fg from total binding. Each point is the mean of duplicates from the same experiment on one cell culture.

mechanism by which the fg molecule is modified was not examined in the present study and will be considered in further investigations.

Estimation of binding affinity. The affinity constant for this interaction was derived from two independent series of experiments. In the first, different concentrations of ^{125}I-fg were incubated with cells in suspension derived from culture of 70,000±6,000 cells/cm^2. Saturation was observed, indicating the presence of a limited number of sites (Fig. 4). Estimation of the dissociation constant K_d from the binding isotherm by a nonlinear regression computer program (24) gave a value of 0.23×10^{-6} M. In another series of experiments, cells in monolayers were used and the K_d was derived from binding competition experiments (25) (Fig. 5 *A*). The binding competition

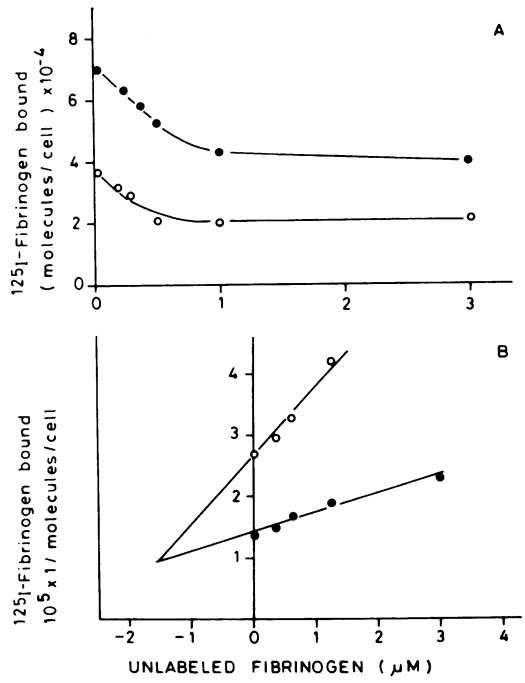

Figure 5. (*A*) Estimate of the affinity of unlabeled fg. (*B*) Dixon plot of binding inhibition data. Confluent human ECs were incubated at 37°C for 30 min with two concentrations of ^{125}I-fg, 0.01 μM (○) and 0.02 μM (●), in the absence or in the presence of increasing concentrations of unlabeled fg. The binding competition data (*A*) were plotted according to Dixon (*B*). Values are means of two replicates from the same experiment.

results were expressed in terms of Dixon plots (Fig. 5 *B*) and yielded an estimated K_d of 1.5×10^{-6} M. These two independent K_d values were comparable when it is considered that correct measurement of binding with cell monolayers is done under nonequilibrium conditions and may give only a rough estimate of the affinity of the ligand for the binding site. As an alternative, cells in suspension may have greater affinity for fg owing to trypsinization or removal from the matrix during detachment of the cells.

Discussion

The results reported here indicate that fg induces EC migration and associates with ECs at specific sites.

The fg-induced EC migration does not appear to be due to degradation products of the molecule or to other contaminating agents. Monospecific Fab fragments directed to fg as well as degradation of fg by plasmin or trypsin destroyed this effect; dialysis of fg, in order to avoid small molecular weight peptides, did not modify the chemotactic activity of the protein. Preliminary observations (data not shown) from ion-exchange chromatography on DEAE cellulose (26) also show that only the fraction associated with fg was able to induce EC migration, making unlikely the possibility that strongly bound low molecular weight material was responsible for this activity. Purified fragments E and D did not induce migration, establishing the requirement for the native molecule. Fg also appears to affect EC migration in a plasmatic milieu. Whereas normal plasma induced EC migration, plasma from an afibrinogenemic patient did not. Addition of fg to afibrinogenemic plasma restored the cellular response. Other proteins known to be associated with the EC matrix in the vessel wall and normally present in plasma, such as fibronectin and von Willebrand factor, induced almost undetectable EC migration. This is compatible with the observation that plasma from an afibrinogenemic patient with a normal content of von Willebrand factor and fibronectin did not stimulate EC migration. Similarly, no migration was observed in the presence of other stimuli known to be chemoattractant for leukocytes, thus confirming the specificity of fg-induced chemotaxis. The present study also shows that fg can specifically associate to ECs. The association of fg to the cells is specific, time dependent, saturable with respect to time and fg concentration, and reversible. Specificity of the binding is indicated by: (*a*) the ability of unlabeled fg but not of other, unrelated proteins such as ovalbumin, myoglobin, and fibronectin to compete for ^{125}I-fg binding; (*b*) the capacity of purified anti-fg Fab fragments to inhibit binding while Fab from nonimmune IgG has no such effect; (*c*) the failure of fg to bind to EUE cells in the same experimental system; and (*d*) the identification of the bound radioactivity as fg-related material on polyacrylamide gel electrophoresis.

Autoradiographic analysis of the electrophoretic display of ^{125}I-material associated to the cells suggests that the molecule is processed during the binding reaction. The cell extract exhibited bands in the positions of the Bβ- and γ-chains with decreased intensity, and an almost complete loss of the Aα-chain. Increased radioactivity was observed at the dye front, suggesting proteolytic degradation of the molecule. The radiolabeled fg displaceable by the cold showed the same digestion of the Aα-chain, but no radioactivity was observed at the dye front.

These modifications of ^{125}I-fg depended on close contact between the molecule and the cells since ^{125}I-fg present in the supernatant and extracted at the end of the incubation with the cells retained all the characteristics of the starting material. Proteolytic digestion of the molecule at the cell membrane may be responsible for the modifications observed. At 4°C, ~30% reduction of unspecific binding was observed. This suggests that part of the unspecifically bound radioactivity is processed or internalized in a temperature dependent way and becomes inaccessible to competition with unlabeled fg. Further investigations are needed to define the exact mechanism by which fg is processed by the cells.

When the cells were used in monolayers, the nonspecific binding of fg was higher (~50% of total binding) than when the cells were used in suspension (~10–15% of total binding). This difference may be associated with a contribution to the nonspecific binding of the ^{125}I-fg bound to the plastic of the petri dish or the extracellular matrix. Alternatively, differences in cell shape and the surface exposed between cells in suspension and in monolayers may account for this difference.

The structural domain within the fg molecule that participates in this interaction remains to be thoroughly characterized. Fragment E competed with fg for binding to the cells, whereas fragment D was ineffective. Since the fragment E used in this study does not interact with fg (16) the competition is probably at the cellular level. This suggests that the amino terminal part of the molecule is involved in this interaction and contrasts with the binding of the molecule with platelets, which specifically involves the D domain (16). Another striking difference between ECs and platelets is that fg binds to apparently unstimulated ECs whereas fg receptor in platelets is inducible. In conclusion, it appears that ECs specifically bind the fg molecule and that the interaction of fg with various cellular systems may have different structural requirements and functional implications.

The data reported here do not provide an answer as to how fg induces migration of ECs; nevertheless, a number of correlates can be identified between association of the molecule to the cells and cell migration: (*a*) Fragment E and Fab fragments directed against fg, which inhibited fg binding to the cells, also inhibited fg-induced cell migration. (*b*) The concentrations of fg active on EC migration are of the same order of magnitude as those associated with binding. (*c*) Saturation was observed for both binding and migration induced activity.

The phenomenon of haptotaxis should be also mentioned. It is possible that fg binds to the filter separating the two compartments of the Boyden chamber and that the cells migrate along a gradient of immobilized fg. This would imply that both binding to the cells and to the filter is required for efficient migration. This possibility could not be excluded even when, under our experimental conditions, the binding of fg to the filter was very low (0.2–0.3% of the total added), and when reducing it further to 0.06–0.08% by soaking the filter with 4% albumin solution did not reduce cell migration significantly (data not shown).

The relevance of these observations to EC behavior in vivo remains to be established. Other authors showed association of fg with vascular endothelium in an experimental system in vivo (27).

The observation that the ECs migrate from lower to higher concentrations of fg is compatible with in vivo and in vitro observations that, when fibrin is present, EC mobility is increased (1, 4). It is likely, therefore, that whatever property is involved in fibrin stimulation of EC migration and proliferation is contained in the native molecule. The partial modification of the molecule by the cells with the disappearance of the Aα-chain may have some implications for the mechanism by which fg associated with and induced migration of ECs, and this merits further investigation.

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