Fibrinogen Induces Adhesion, Spreading, and Microfilament Organization of Human Endothelial Cells In Vitro

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Abstract. Human umbilical vein endothelial cells (ECs) have been shown to attach to a substratum of fibrinogen (fg). Later, ECs undergo spreading, organization of thick microfilament bundles of the stress fiber type, and formation of focal contacts (adhesion plaques) that correspond to accumulation of vinculin at the cytoplasmic aspect of the ventral membrane. The rate of attachment to fg and the type of spreading is virtually identical to that obtained on substrata coated with fibronectin (FN). Antibodies to fg, but not to FN, prevent EC adhesion to fg; conversely, antibodies to FN, but not to fg, prevent adhesion of ECs to a FN-coated substratum. The removal of residual FN contamination from fg preparations by means of DEAE-cellulose chromatography does not result in any difference in EC adhesion on fg. Moreover, pretreatment of cells with inhibitors of synthesis and release of proteins does not impair their adhesion capacity on

an fg-coated substratum. In contrast, human arterial smooth muscle cells do not adhere and spread on fg substrata but do so on FN. The synthetic peptides (Gly-Arg-Gly-Asp[GRGD] and Gly-Arg-Gly-Asp-Ser-Pro[GRGDSP]) containing the tripeptide Arg-Gly-Asp (RGD), originally found to be responsible for the cell binding activity of FN, have been found to inhibit EC spreading and the redistribution of their cytoskeleton, including the formation of stress fibers and the localization of vinculin either on fg or on FN. Conversely, the synthetic peptide Arg-Gly-Gly (RGG) was completely uneffective in inhibiting the adhesion and the sequence of events leading to spreading and cytoskeletal organization. These results indicate that ECs, but not smooth muscle cells, specifically adhere and spread on an fg substratum and this occurs by recognition mechanisms similar to those reported for FN.

ENDOTHELIAL cells (ECs)[1] provide the vascular system with a renewable nonthrombogenic surface. Factors that contribute to the adhesion, motility, and growth of ECs are important mediators for the maintenance of vascular integrity. Many authors (20, 32, 43, 51) have shown that the EC extracellular matrix is able to influence several aspects of cellular behavior, including attachment, spreading, and migration, and may in part initiate and control vascular response in growth, differentiation, wound repair, and neoplasia.
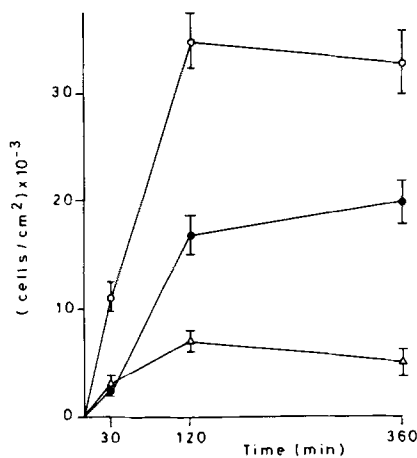
The basement membrane of ECs is composed of numerous macromolecules such as collagen(s), fibronectin (FN), laminin, and glycosaminoglycans (24). Besides these molecules, the other substratum that ECs might be expected to interact with is fibrinogen (fg) and/or fibrin. At sites of vascular injury fg/fibrin tend to accumulate on the surface of the exposed subendothelium (4, 44) and might constitute a substratum over which ECs can attach, spread, and proliferate.

It has been shown in vitro and in vivo that ECs can migrate and proliferate into a fibrin clot and that fg and its derivatives may greatly influence EC functions (7, 9, 28, 37, 42, 47). Recently we have shown that native fg specifically binds to ECs and induces their migration (8).

The microfilamentous cytoskeleton of ECs is organized in thick bundles (stress fibers) that may be visualized both in vitro and in situ (13, 41, 49), and that have been postulated to be contractile (for review see reference 22). The distribution of stress fibers in situ seems to be influenced by a number of different factors (49), including hypertension (13), and is particularly prominent during regeneration of the injured vessel wall (14, 26). Moreover, EC stress fibers have been shown to attach to the cytoplasmic aspect of the plasma membrane in situ at structures resembling typical adhesion plaques and to correspond to fibrillar bundles of matrix molecules (26). The organization of such attachment sites may be particularly relevant in the process of re-endothelialization of the intimal layer (26).

In this report we show that substratum-bound fg, like to FN, is able to promote in vitro EC adhesion, spreading, organization of thick microfilament bundles, and formation of

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1. *Abbreviations used in this paper:* ECs, human umbilical vein endothelial cells; fg, fibrinogen; FN, fibronectin; IRM, interference reflection microscopy; M199, medium 199; R-PHD, rhodamine-labeled phalloidin; SMCs, human arterial smooth muscle cells; VN, vitronectin.

Figure 1. Time course of EC attachment to FN- (*open circle*), fg-(*solid circle*), or BSA- (*open triangle*) coated glass. The coverslips were coated with 10 μg/ml FN, 250 μg/ml fg, 1 mg/ml BSA before the assay. [³H]Thymidine-labeled cells were seeded onto different substrata. After the indicated periods of incubation the cells were washed twice with PBS and the cells bound to the substrata extracted and counted. The values are mean ± SEM of three experiments performed on three separate cell cultures.

focal contacts (adhesion plaques), including local assembly of vinculin molecules. This chain of phenomena is prevented by the presence in the incubation medium of a peptide containing the sequence RGD, which has been shown to be part of the cell-binding domain of several matrix molecules (25, 38) and also of fg (15, 23, 40). In contrast, human arterial smooth muscle cells (SMCs) fail to adhere and organize their microfilaments on fg substrata.

The findings reported in this paper may be relevant for the mechanism of repair of vascular lesions.

Materials and Methods

Cells

ECs from human umbilical cords were obtained and cultured as described previously (1). The cells were grown to confluence on plastic flasks (Falcon Labware, Oxnard, CA) in medium 199 (M199) supplemented with 20% FCS. The cells were cultured at 37°C in a water-saturated atmosphere of 95% air-5% CO_2 and fed three times a week. The experiments reported in this paper were carried out with ECs at the first passage in vitro. ECs were characterized by indirect immunofluorescence using rabbit anti-human factor VIII antigen (Behringwerke AG, Marburg, Federal Republic of Germany) (1). SMCs were kindly donated by Dr. G. Gabbiani (University of Geneva, School of Medicine, Geneva, Switzerland). The cells were grown in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum and cultured as described for ECs. All culture reagents were from Gibco Laboratories (Paisley, Scotland).

Purification of FN, fg, and fg Derivatives

Human plasma FN was purified from freshly drawn citrated blood plasma by affinity chromatography on gelatin-Sepharose (10).

Human fg was purified according to described procedures (29). Polyacrylamide gel electrophoresis analysis (30) of the purified material in the presence of SDS and reducing agents demonstrated intact α-, β-, and γ-chains. Plasminogen, factors II, XIII, von Willebrand factor, and fibrin monomer could not be demonstrated in any preparation by established criteria (8). Contaminating FN was lowered by affinity chromatography on gelatin-Sepharose to 5 ng/mg as evaluated by solid-phase radioimmunoassay (10). To eliminate contaminating FN, fg was further chromatographed on DEAE-Sephacel columns (2.5 × 40 cm) after dialysis against the starting

buffer (11). A linear ionic strength and pH gradient was applied with a gradient maker (LKB Instruments, Inc., Bromma, Sweden) using a starting buffer of 0.04 M Tris, pH 8.5, and a final buffer of 0.1 M Tris, pH 5.5, + $CaCl_2$ 0.1 M. Two distinct protein peaks were resolved (peak I and II). The pooled fractions were dialyzed against NaCl 0.5 M, Tris HCl 0.05 M, pH 7.6.

SDS PAGE according to Laemmli (30) of the reduced starting material showed α-chain heterogeneity, intact β- and γ-chain, and a band (215,000 mol wt) representing the polypeptide chains of FN. The electrophoretic pattern of peak I was similar to that of the starting material, except that there were no bands in the position corresponding to FN, while peak II showed a large band corresponding to FN. Each peak was tested for FN concentration by solid-phase radioimmunoassay (10). FN was undetectable in peak I (<1 ng/mg fg) and was 17 ng/mg fg in peak II.

Synthetic Peptides

Peptides (GRGD, GRGDSP, RGG) were synthesized at Sanofi Research (Montpellier, France) by solid-phase synthesis. The purity of these peptides was assessed by high performance liquid chromatography. Hydrolysates (24 h) of the peptides were subjected to amino acid analysis on an automated amino acid analyzer. The peptides were more than 90% homogeneous.

Antibodies

Rabbit anti-human plasma fg or FN IgGs were prepared as described (34). To ensure specificity, the antisera to fg were adsorbed with plasma from an afibrinogenemic patient, and the antisera to FN with plasma was FN-deprived by affinity chromatography on gelatin-Sepharose. The IgG fractions were isolated from the antisera to fg and hydrolyzed with papain. The Fab fragments were immunochemically purified by affinity chromatography on fg-Sepharose. The specificity and affinity of these Fab fragments have already been detailed. Typically, the preparation contains Fabs directed to major structural domains of the native molecule, namely D and E (34). Sephadex G150 chromatography and SDS PAGE verified the absence of non-hydrolyzed IgGs and Fc fragments in the Fab preparations. Nonimmune Fab fragments were isolated by carboxymethyl-cellulose chromatography of papain digest of rabbit IgG. The antibodies were checked by immunodiffusion for the lack of cross-reactivity against the alternative antigen, e.g., anti-FN activity in the anti-fg antibodies and vice versa. The vitronectin (VN) antibody raised in the rabbit was obtained from Behringwerke. The antibody was tested for its ability to inhibit EC adhesion to human purified VN (Behringwerke). EC adhesion to VN-coated (30 μg/ml) glass was measured with the same procedure used for fg- or FN-coated substrata described below in details. The antibody was then used at a concentration able to inhibit cell adhesion to VN substrata by 70 ± 2%. Polyclonal antibody raised against platelet glycoprotein (GP) IIb-IIIa (R343) was a kind gift of Dr. E. Plow and M. H. Ginsberg (Research Institute of Scripps Clinic, La Jolla, CA). This antibody has been shown to precipitate GP IIb-IIIa–related antigens in EC, with a mobility similar to platelet GP IIb-IIIa by polyacrylamide gel analysis (39).

Preparation and Coating of Glass Coverslips

Glass coverslips (10 mm in diameter) were cleaned by sonication for 10 min, then immersed in ethanol/ether (1:1 vol/vol) for 2 h and dried before placing in multiwell tissue culture plates (Falcon Labware). The coverslips were coated either with 0.3 ml FN, fg, DEAE-Sephacel elution peaks I or II at the required concentrations, or with 0.1% BSA in PBS containing Ca^{++}-Mg^{++}, pH 7.4, for 2 h at 37°C. Residual protein-binding sites were blocked by further incubation (30 min, 37°C) with 0.2% BSA in PBS containing divalent ions. In some experiments, gelatin (type III; Sigma Chemical Co., St. Louis, MO) was used at 1% concentration in 0.3 ml water and incubated with the coverslips overnight at 4°C. Coverslips were washed twice with PBS before using for experiments.

To evaluate the actual amount of coverslip-bound protein, fg was trace-labeled with carrier-free ^{125}I by the modified chloramine T method previously described (35). Bound radioactivity was measured by scintillation spectrometry.

Adhesion and Spreading Assays

ECs grown in 25-cm² flasks (~1 × 10⁶ cells/flask) were incubated in 3 ml M199 containing 10 μCi/ml of [³H]thymidine (specific activity 5 Ci/mmol; Amersham International, Amersham, England) for 24 h. The cells were then washed with 5 ml PBS and detached by brief exposure (1-2 min) to
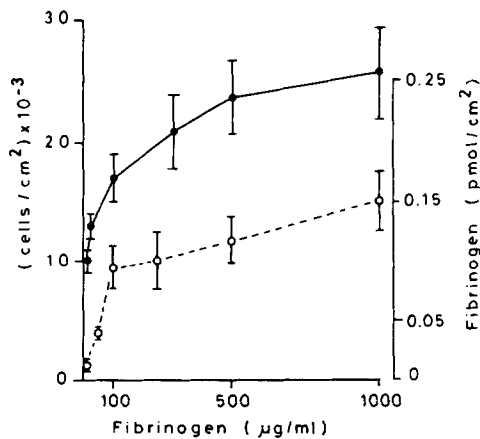
Figure 2. EC attachment (*solid circle*) to fg-coated coverslips as a function of fg concentration. Coverslips were incubated with different concentrations of fg for 2 h at 37°C, then washed twice with PBS. [³H]Thymidine-labeled ECs were seeded onto fg-coated glass. After 2 h incubation, cells were washed twice with PBS and substratum-bound cells extracted and counted. The values are mean ± SEM of three experiments performed on three separate cell cultures. Fg concentration on the coverslips (*open circle*) was evaluated by coating the glass with [¹²⁵I]fg as described in Materials and Methods. The values are means ± SEM of four separate assays.

a prewarmed 0.25% trypsin plus 0.02% EDTA solution in PBS. The trypsin action was stopped by adding 10 ml of M199 containing 20% FCS. The cells were immediately spun at 1,200 g and resuspended in M199 without serum at a concentration of 80–100,000 cells/ml and 1 ml of cell suspension was seeded in each well. After 2 h incubation at 37°C, nonadherent cells were washed away by rinsing twice with PBS and adherent cells were solubilized by overnight incubation in 0.3 ml Soluene-350 (Packard Instrument Co., Downers Grove, IL) and counted by liquid phase scintillation spectrometry. In some experiments, adherent cells were characterized as ECs by means of indirect immunofluorescence using factor VIII antigen antibodies (see above). In some experiments, in order to prevent de novo protein synthesis, 20 μM cycloheximide (Sigma Chemical Co.) was added to cells 60 min before detachment from flasks and kept in all buffers used, even during the adhesion assay. In a further set of experiments, 1 μM monensin (Sigma Chemical Co.) was also added to buffers before and during the adhesion assay to prevent the secretion of adhesive factors.

The direct involvement of different molecules in promoting the adhesion of ECs was also tested by exposing substrata and cells to specific antibodies. Anti-FN, anti-VN, anti-fg Fab fragments, and preimmune rabbit IgG Fab fragments were added to coated coverslips for 1 h at 37°C before beginning the adhesion assay. In the same experiments the antibodies were also added to cell suspensions for 30 min before seeding cells into the culture wells and kept during the adhesion assay. In some experiments the effects of synthetic peptides were evaluated by means of a spreading assay. This was done by counting rhodamine-labeled phalloidin (R-PHD)–stained cells (see next paragraph) and scoring as spread ECs those in which well-defined microfilament bundles of the stress fibers type were observed as the percentage of total adherent cells.

Fluorescence Microscopy

Coverslip-attached cells were fixed in 3% formaldehyde in PBS, pH 7.6, containing 2% sucrose for 5 min at room temperature. After thorough rinsing in PBS, cells were made permeable to large molecules by soaking coverslips for 5 min in Hepes-Triton X-100 buffer (20 mM Hepes, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, and 0.5% Triton X-100). This fixation and permeabilization procedure has been shown to yield optimal preservation of cells for adhesion studies (33, 45).

Fixed and permeabilized cells were stained with 2 μg/ml R-PHD (a kind gift of Dr. T. Wieland, Max Planck Institute for Experimental Medicine, Heidelberg, Federal Republic of Germany) for 30 min at 37°C and mounted in 50% PBS-glycerol. R-PHD has been shown to bind firmly to F-actin (50).

In some experiments coverslip-attached cells, fixed and permeabilized as above, were simultaneously stained for F-actin with fluorescein-labeled phalloidin and with a mouse monoclonal antibody against vinculin (code

Table I. EC Attachment to fg and FN Substrata and Effect of Antibodies

Substrata	Cells/cm²
BSA	9,100 ± 1,400
Fg	16,800 ± 500
Fg-αFN	18,200 ± 1,600
Fg-αfg	1,900 ± 100
Fg-αVN	21,100 ± 2,900
FN	34,600 ± 4,000
FN-αFN	12,000 ± 3,400
FN-αfg	35,900 ± 700
Fg DEAE Sephacel-fraction I	17,900 ± 2,000
Fg DEAE Sephacel-fraction II	16,500 ± 1,800
Fg DEAE Sephacel-fraction I and FN	17,000 ± 700

ECs were seeded onto coverslips coated with BSA (1,000 μg/ml), fg (250 μg/ml), FN (10 μg/ml), fg-DEAE-Sephacel fractions I and II (250 μg/ml) or fg-DEAE-Sephacel fraction I added with 0.5 μg/ml FN. The antibodies against fg, FN, or VN were added to protein-coated coverslips for 1 h before the assay and were maintained during the assay. The values are expressed as number of cells attached in respect to the total number of cells seeded. The values are means ± SEM of three experiments performed on three separate cell cultures.

6501, clone VIN-11-5; Bio-Yeda, Rehovot, Israel). The vinculin localization was revealed with a rhodamine-tagged rabbit anti-mouse IgG (DAKO-PATTS, Copenhagen, Denmark).

Observations were carried out in a Leitz Diavert microscope equipped for epifluorescence and interference reflection microscopy (IRM). Fluorescence images were recorded on Kodak TriX films exposed at 1000 ISO and developed in Nucleol BF 200 (Chimifoto Ornano, Milan, Italy); parallel IRM patterns were recorded on Ortho 25 films developed in Neuton NEUTRAL (Agfa, Gevaert, Milan, Italy).

Results

Attachment of ECs to fg

Suspensions of ECs were seeded on glass coverslips coated with different proteins (see Materials and Methods). As shown in Fig. 1, more than 30,000/cm² cells attached within 2 h to FN, ~17,000 to fg-coated glass coverslips, and fewer (7,000) adhered to a BSA substratum. The endothelial nature of adherent cells was established by their positivity for the presence of factor VIII antigen. After 2-h adhesion tests, the nonadherent cells showed more than 95% viability by Trypan blue exclusion test.

The kinetics of the process appeared to be very similar for both FN and fg, since it reached a plateau in ~2 h.

The degree of EC adhesion was also dependent on the concentration of fg and reached a plateau at 100–500 μg/ml (Fig. 2). Then the conditions routinely used for assays of EC adhesion to fg included coverslip coating with 250 μg/ml fg and 2 h incubation. Using [¹²⁵I]fg, we evaluated the amount of the protein bound to the glass after the coating procedure (Fig. 2). The curve of fg binding to the coverslips was parallel to that of the adherent cells. The amount of [¹²⁵I]fg bound to the glass did not significantly change between 100 and 500 μg/ml; a moderate increase was observed using 1,000 μg/ml. We routinely adopted an FN concentration of 10 μg/ml, since the number of adherent cells did not vary between 10 and 250 μg/ml (data not shown).

Role of Contaminating FN on EC Adhesion to fg

To investigate whether EC adhesion to fg was in fact attributable to this molecule and not to FN contaminating the
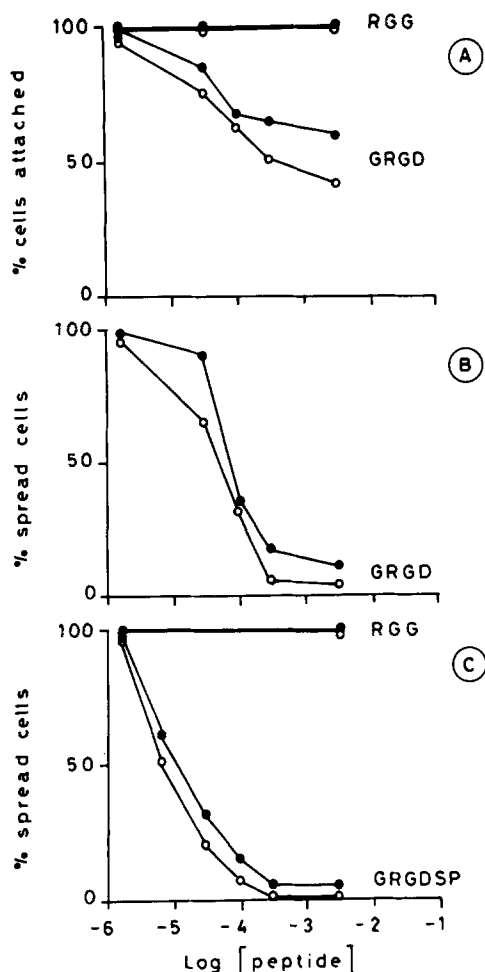
Figure 3. Concentration-dependent inhibition of EC adhesion (*A*) and spreading (*B* and *C*) to FN or fg by synthetic peptides. For adhesion experiments [^{3}H]thymidine-labeled ECs were seeded onto FN (10 μg/ml, *solid circle*), fg (250 μg/ml, *open circle*) in the presence of increasing concentrations of peptides. The values, expressed as percentage of cells attached to different substrata in the absence of peptides, are means of three separate experiments performed on three distinct cell cultures. SEM never exceeded 15% of the mean. The cells attached in the absence of peptides were 38,190 ± 3,100/cm² on FN, 27,156 ± 1,930/cm² on fg (means ± SEM of triplicate experiments). For spreading experiments cells were fixed and stained with R-PHD as described in Materials and Methods. The number of spread ECs showing well-defined microfilament bundles was expressed as percentage of total adherent cells. Values are means of three separate experiments. SEM never exceeded 10% of the mean.

preparation, a series of experiments was performed using different approaches.

Specific FN antibodies were incubated with fg-coated coverslips and ECs and left in the culture medium for the duration of the experiment. As shown in Table I, no change in EC adhesion to fg was observed in these conditions. However, the antibody concentration used was able to reduce EC adhesion to FN by ~60%.

The FN concentration contaminating the fg preparation used in these experiments was removed by affinity chromatography on Sepharose-bound gelatin. To remove residual minimal amounts of contaminating FN, a chromatographic

fractionation of fg on DEAE-Sephacel was performed by continuous salt and pH gradient elution as previously described in detail (11). The elution pattern revealed two peaks of fg differing for elution properties.

The FN amount contained in the two fractions was <1 ng/mg fg in fraction I and 17 ng/mg fg in fraction II. When the coverslips were coated with either fraction I or II no difference in EC adhesion was observed (Table I). Furthermore, addition of 0.5 μg/ml of FN to peak I did not change EC adhesion as well.

To exclude that VN, possibly contaminating the fg preparation used, could mediate EC adhesion, a specific VN antibody was tested. As reported in Table I, cell adhesion on fg was unaffected.

Role of Protein Synthesis and Secretion on EC Adhesion to fg

To investigate the role of synthesis and secretion of proteins in the process of adhesion to fg, ECs were treated with cycloheximide and monensin. Cycloheximide (20 μM) was added to cells 60 min before detachment. At this concentration, protein synthesis was inhibited by 98% as previously shown (45). Monensin (1 μM) was used to prevent secretion of adhesive factors (46) and was also added to all buffers used and to the adhesion medium.

Treatment of cells with cycloheximide and monensin, either individually or in combination, did not significantly reduce cell adhesion to BSA, fg, or FN (data not shown).

Effects of Hirudin and Aprotinin on fg-induced EC Adhesion

To investigate whether thrombin or plasmin might play any role in adhesion of ECs to fg, hirudin (10 U/ml) and aprotinin (4,000 KIU/ml) were added to the medium during the adhesion experiment. No effect of these substances was observed on EC adhesion to fg, since 16,800 ± 400 of cells adhered in the absence of inhibitors, 16,950 ± 401 in the presence of hirudin, and 17,420 ± 901 in the presence of aprotinin (means ± SEM of four experiments). The concentrations of inhibitors used were able to neutralize ~1–10 U/ml thrombin and 4,000 U/ml plasmin, respectively.

Inhibition of EC Adhesion and Spreading by Synthetic Cell Attachment-promoting Peptides

To find out whether cell adhesion to fg was mediated by the same molecular mechanism as to FN, the synthetic peptide GRGD, containing the tripeptide RGD, originally found to be responsible for the FN cell attachment activity (38), was tested on ECs. The RGD sequence is also found at residues 95–97 and 572–574 of the 611 amino acids of the α-chain of fg. As shown in Fig. 3 *A*, GRGD was able to inhibit EC adhesion either to fg or to FN. In contrast, GRGD was completely uneffective up to 1 mg/ml in inhibiting EC adhesion to gelatin (30,412 ± 3,200 cells/cm² in the absence and 31,500 ± 1,200 cells/cm² in the presence of the peptide). When the peptide RGG was used to test the structural specificity of the GRGD effect, no inhibition of EC adhesion was observed, both on FN and fg substrata.

Fig. 3, *B* and *C*, reports the effects of GRGD and GRGDSP on EC spreading. Both peptides had a comparable activity in preventing cell spreading on fg and on FN; a virtually

complete inhibition of cell spreading was obtained in both cases, whereas, by simply considering the adhesion phenomenon, only 50% inhibition was achieved by GRGD.

We tested whether an antibody raised against platelet GP IIb-IIIa inhibited EC spreading on fg. This antibody (R343) has been shown (39) to precipitate GP IIb-IIIa–related antigen in ECs with a mobility similar to platelet GP IIb-IIIa by polyacrylamide gel analysis. While EC adhesion was inhibited by 33% (1,712 ± 150 vs. 1,329 ± 102), cell spreading was blocked by the presence of the antibody (94% of total adherent cells spread in the absence, while only 2% spread in the presence of the antibody).

Spreading and Organization of Microfilamentous Structures of ECs Plated on fg

The distribution of the microfilamentous cytoskeleton and the localization of vinculin were studied by fluorescence microscopy on fixed and permeabilized ECs. F-actin was revealed with fluorochrome-labeled PHD, a fungine cyclic polypeptide that binds selectively to F-actin (50). Vinculin was identified immunologically with a specific monoclonal antibody that has been shown to recognize the mammalian form (18). In the same cells, the extent of adhesion was evaluated by IRM, a technical means that records the distance between the adhesion substratum and the cell ventral membrane (5, 27, 48): the smaller the distance, the darker the signal intensity, black indicating tight adhesion.

When ECs were plated on BSA-coated glass coverslips they remained rounded and only a small proportion partially adhered to the substratum. In these conditions, F-actin did not assemble in microfilament bundles after 30 min (Fig. 4 A); at 2 h after plating only a few cells showed thin microfilament bundles and none exhibited thick bundles of the stress fiber type (Fig. 4 B). No adhesion plaques were apparent by IRM (not shown).

Upon plating on FN- or fg-coated glass coverslips, ECs progressively adhered and spread. In a few cells attachment was apparent already at 30 min but no obvious microfilament bundles of the stress fiber type were visible (Fig. 4, C and E). At 2 h (Fig. 4, D and F) spreading of most cells was complete and a network of thick microfilament bundles had formed. The microfilamentous cytoskeleton was stable for at least 1 d and its pattern was identical to that shown at 2 h (not shown). No obvious difference in microfilament distribution was found in ECs plated either on FN or on fg.

At 2 h after plating, spreading and cytoskeleton organization (Fig. 4 G) was also complete when coverslips were coated with DEAE-Sephacel peak I fraction, which has been shown to be devoid of significant FN contamination. This rules out the possibility that spreading activity may be attributed to contaminant FN. Conversely, spreading and stress fiber assembly was totally abolished when ECs were seeded on peak I–coated coverslips in the presence of GRGD (Fig. 4 H). The control peptide RGG by no means impaired spreading and cytoskeletal organization (not shown).

The formation of stress fibers occurred simultaneously with the appearance of vinculin streaks at their endings in correspondence with adhesion sites. In fact, cells plated either on fg (Fig. 5, A–C) or on FN (Fig. 5, G–I) showed multiple IRM black streaks, which mostly corresponded to the endings of stress fibers and distinct arrowhead-shaped vinculin speckles (e.g., at arrowheads). Again, no obvious difference was appreciated in cells spread either on fg or on FN. While in the presence of GRGD the formation of tight adhesion sites was abolished (Fig. 5, D–F).

Spreading and Organization of Microfilamentous Structures of SMCs Plated on fg

The organization of microfilaments has also been studied on fixed and permeabilized SMCs seeded on BSA, fg, and FN. Fig. 6 shows that on both BSA and fg, in the absence of serum, most cells were void of organized microfilament bundles (A and B). In contrast, SMCs seeded on FN showed a well-developed array of stress fibers, indicating that the cells had undergone full spreading (Fig. 6 C). Of the total adherent SMCs, 90% spread on FN while only ∼2% on fg or BSA.

This data indicate that spreading on fg does not occur on SMCs, showing that, among cells of the vessel wall, such an event is peculiar to ECs.

Discussion

The results reported here indicate that ECs adhere to an fg substratum and eventually undergo spreading, expression of stress fibers, and formation of adhesion plaques. The activity of fg in supporting such phenomena is somehow weaker than FN, since the total number of adhering ECs was always lower. Qualitatively, however, the pattern of microfilament organization and number and extent of focal adhesion contacts are closely comparable. This phenomenon is of particular relevance, since it provides evidence that the plasma membrane has interacted via specific receptors with the extracellular matrix (for review see reference 6; see also reference 19) and that this interaction has triggered a cascade of events leading to the organization of the adhesion plaque at which vinculin and other proteins are associated (2, 3, 16–18, 48).

Grinnell et al. (21) reported that baby hamster kidney cells did not attach to substrata composed of purified fg, but that adhesion was supported when plasma FN was covalently cross-linked to fg. The data reported here do not confirm this observation on ECs.

The ability of fg does not appear to be due to contaminating FN or VN, since addition of FN or VN antibodies to the culture medium or removal of residual contaminating FN from fg preparations by DEAE-Sephacel chromatography does not impair fg-induced EC adhesion and spreading. Other possible contaminants, like plasminogen, factor II, XIII, and VIII, von Willebrand factor, and fibrin monomers could not be demonstrated in any preparations by established criteria (8) and do not appear, therefore, to play any major role in fg-induced EC adhesion.

ECs have been shown to interact with fibrin (28) and fg plasmin-degradation products (7). These molecules have been shown to induce EC contraction and disruption of the cell monolayer (7, 28). Therefore, we tested the possibility that thrombin or plasmin, possibly generated in the system, might interact with fg or directly with ECs to modulate the adhesion process. The results indicate that very high concentrations of hirudin and aprotinin, capable of inhibiting high concentrations of thrombin and plasmin, do not virtually
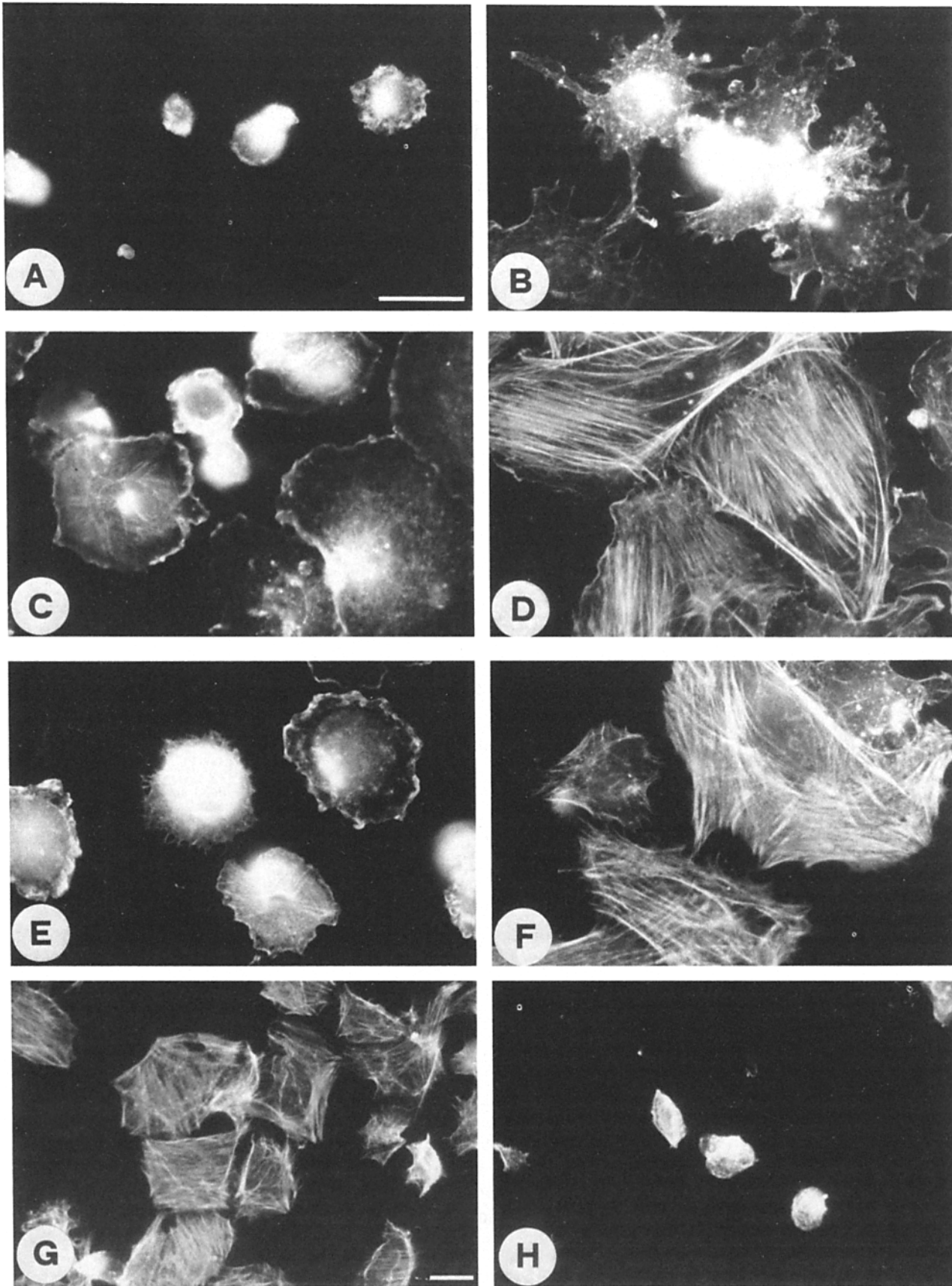
Figure 4. Distribution of F-actin stained by R-PHD in ECs seeded on coverslips coated with BSA (*A* and *B*), fg (*C* and *D*), FN (*E* and *F*), and DEAE-Sephacel peak I (*G* and *H*). Time after seeding was 30 min (*A, C,* and *E*) and 2 h (*B, D, F, G,* and *H*); in H incubation was carried out in the presence of 0.6 mg/ml GRGD. No microfilament bundles of the stress fiber type appear 30 min after seeding on
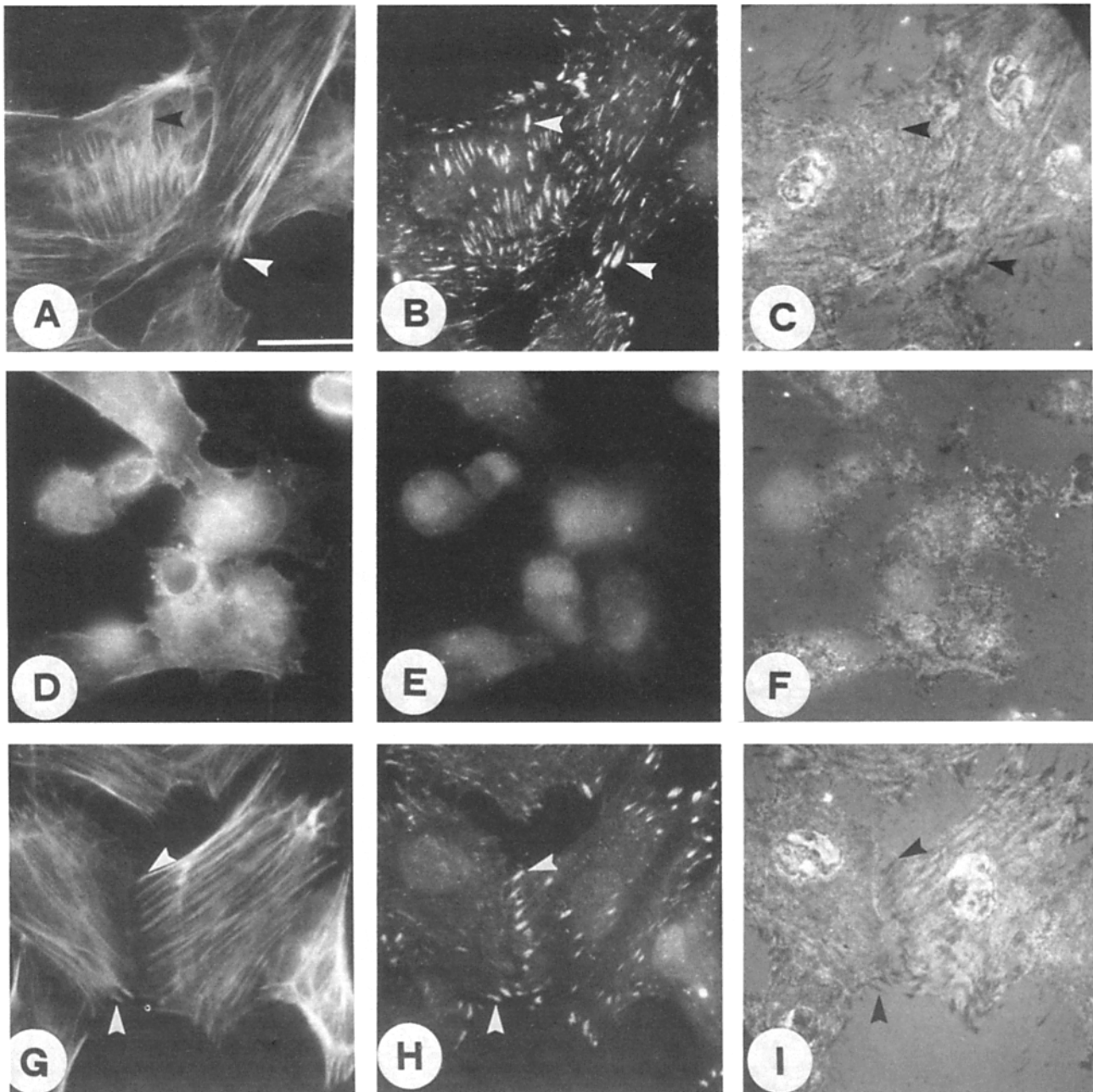
Figure 5. Immunofluorescence localization of vinculin (*B, E,* and *H*) and IRM patterns of ECs (*C, F,* and *I*) compared with the distribution of fluorescein-labeled phalloidin–stained F-actin (*A, D,* and *G*). ECs were allowed to spread for 2 h on fg-coated coverslips in the presence (*D–F*) or in the absence (*A–C*) of the tetrapeptide GRGD (0.6 mg/ml) or on FN-coated coverslips (*G–I*). The peptide inhibited spreading of ECs on fg as well as the appearance of vinculin speckles and adhesion plaques (*A–C*, e.g., arrowheads). Bar, 5 μm.

change the number of cells adhering to fg and thus exclude the need for these two enzymes in fg-induced EC adhesion and spreading.

EC attachment to fg might be influenced by de novo synthesis and release of FN or other adhesive proteins by the cells. The data reported in this paper do not support this pos-

sibility, since cycloheximide and monensin treatment at concentrations able to suppress synthesis and external exposure of proteins did not affect EC adhesion to fg. In addition anti-FN antisera were uneffective in reducing EC adhesion to fg substrata, thus indicating that membrane-associated FN does not mediate EC adhesion to fg.

BSA, fg, or FN (*A, C,* and *E*). 2 h after seeding microfilament bundles did not appear in ECs plated on BSA (*B*) but were abundantly assembled in cells plated on fg, FN, or peak I (*D, F,* and *G*). When ECs were plated on peak I–coated coverslips and incubated in the presence of the tetrapeptide GRGD for 2 h, spreading and microfilament organization were impaired (*H*). No obvious difference in microfilament distribution was found in ECs after spreading, either on fg (*D*) or on FN (*F*). Bars, 5 μm.
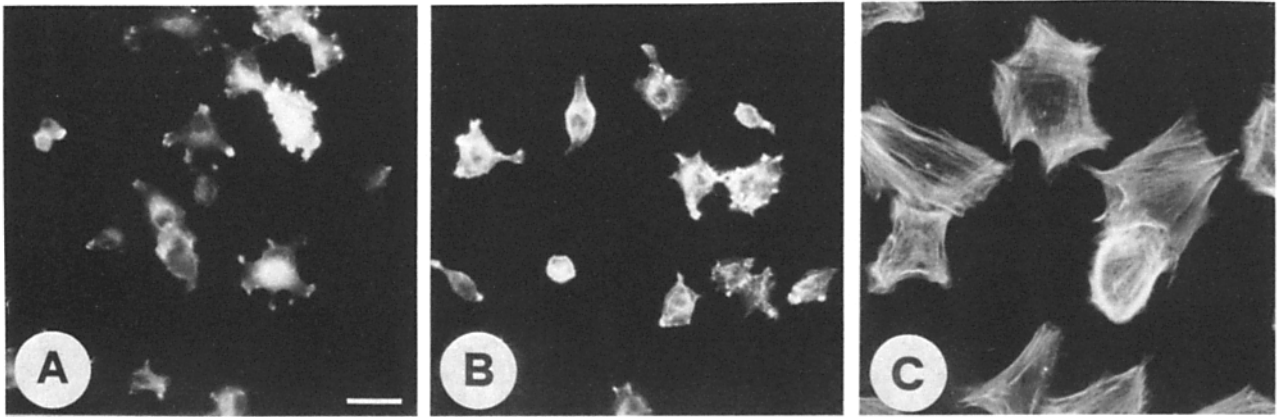
Figure 6. Distribution of F-actin after staining with R-PHD in SMCs seeded on coverslips coated with BSA (*A*), fg (*B*), or FN (*C*) 2 h after seeding. Bar, 5 μm.

The structural domain of the fg molecule that takes part in EC adhesion remains to be fully characterized. Piersch-bacher and Ruoslathi (38) demonstrated that the ability of FN to bind to cells and to promote adhesion can be accounted for by the tripeptide RGD located in the cell attachment domain of FN. This sequence is also present in several other proteins including fg (38). These authors showed that peptides containing the RGD sequence inhibited the attachment of various cell lines to FN and vitronectin, while they did not have any effect on cell attachment to collagen (25). Plow et al. (40), Haverstick et al. (23), and Gartner and Bennett (15) demonstrated that RGD-containing peptides inhibited the binding of FN and fg to stimulated platelets and thus established a common feature in the interaction of either molecule with platelets. In this paper we show that GRGD and GRGDSP block EC spreading and the organization of micro-filaments on fg and FN. ECs might therefore recognize a similar molecular domain of fg and FN as do fibroblasts or platelets. However, while the GRGD sequence is poorly active on platelets (23, 40), ECs appear to equally recognize both GRGD and GRGDSP. In the presence of GRGD, a relative discrepancy between data of adhesion versus spreading has been observed. This peptide, in fact, could never fully inhibit adhesion, while almost complete inhibition of spreading was achieved. This result points to the idea that a trans-membrane cascade of events leading to cytoskeletal reorganization depends upon an RGD recognition mechanism.

The GRGD effect was evident only when cells were plated either on FN or on fg but not on gelatin. Hyman et al. (25) reported that RGD-containing peptides did not prevent normal rat kidney cell attachment to collagen; the author suggested that, even if collagen contains many RGD sequences, they may not be the recognition sites for cells during the initial stage of attachment.

Several reports (12, 31, 36, 39) showed that proteins immunologically related to the platelet fg receptor GP IIb–IIIa are also present in ECs. In preliminary experiments we observed that an antibody against the platelet GP complex was able to block EC spreading on fg; this suggests that the cell binding sites for fg on ECs is immunologically related to platelet GP IIb–IIIa.

In conclusion, the results reported here indicate that ECs adhere to fg as they do to FN. The ability of RGD-containing peptides in inhibiting cell spreading on either molecule suggests that, in analogy with platelets, EC interaction on these substrata is mediated by the same recognition determinant.

The relevance of these observations to the behavior of ECs in vivo remains to be established. Fg and fibrin are known to interact with subendothelial structures and to accumulate at sites of vascular injury (4, 44). ECs at the periphery of the lesion might therefore be expected to come in contact with fg and fg derivatives. Indeed, the minimal fg concentration able to promote EC adhesion and spreading is less than one-tenth of that of plasmatic fg and might therefore be easily reached at sites of vessel injury.

It has been reported by several authors that extracellular matrix molecules deeply affect EC spreading, migration, and proliferation (32, 51). Fg appears to bind specifically to ECs (8), to promote adhesion and organization of microfilaments, and, in the Boyden chamber system, it seems to induce migration of these cells. Further investigations are needed to establish whether the above fg-induced activities have any relevance in the rate at which ECs migrate into a vascular lesion and affect intimal repair.

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