The Mechanism of Fibrin-Induced Disorganization of Cultured Human Endothelial Cell Monolayers

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Deposition of polymerizing fibrin on the vascular endothelium is the final event in intravascular coagulation. Exposure of fibrin clots to confluent monolayers of cultured human endothelial cells for 4 to 24 hours resulted in the disappearance of their normal cobblestone morphology and in the formation of endothelial cell aggregates. The present study was designed to evaluate the conditions and structural requirements of the fibrin clot for the induction of disorganization. Even after harsh treatment with denaturing agents or loading with large amounts of fibrinogen antibodies, polymerized fibrin always induced disorganization of the monolayers. In contrast, soluble fibrin that was kept in solution by either fibrinogen, fragment D-cate, or the tetrapeptide Gly-Pro-Arg-Pro did not cause any alteration of the monolayers. The fibrinogen degradation product D-cate (M₄, 94,000) itself had no microscopically detectable influence on the monolayer structure. In the absence of fibrin, the effect of thrombin on endothelial cells was found to be distinct from that induced by fibrin; however, the exposure of pieces of glass coverslips caused alterations in morphology indistinguishable from the fibrin-induced disorganization of the monolayer. Experiments using protein-coated polyester films indicated that the ability of the endothelial cells to attach to the overlying material, independent of its chemical structure, is the prerequisite for the induction of disorganization, but not a defined component of the fibrin molecule. Disorganization of vascular endothelium in vivo might be important for the organization and revascularization of an occluding thrombus. (Arteriosclerosis 6:139–145, March/April 1986)

The vascular endothelium contributes anticoagulant as well as procoagulant activities to the regulation of the coagulation system. A nontrombogenic surface and the expression of fibrinolytic activities by the endothelial cells guarantee the patency of the vascular tree. Surface-bound heparan sulphate, which accelerates the inactivation of thrombin by enhancing the formation of the antithrombin III/thrombin complex, and the membrane cofactor thrombomodulin, which facilitates the activation of protein C in conjunction with thrombin, are important surface-mediated mechanisms of the vessel wall that are anticoagulant in nature. In addition, the synthesis and release of prostacyclin and of tissue plasminogen activators account for the inhibition of platelet activation and the continuous lysis of small amounts of fibrin. On the other hand, the synthesis of factor VIII:Ag, specific binding of factors IX/IXa and X/Xa, and the induction of tissue factor production in the endothelium imply an involvement of the vascular endothelial cells in local thrombus formation. Various states of disease may disturb the subtle balance between the pro- and anticoagulant forces and may subsequently lead to generalized or local deposition of fibrin on the endothelial cell surface.

While the effect of fibrin degradation products on cultured endothelial cells has been studied in detail, little is known about the effect of fibrin on the vascular endothelium. Transmission electron microscopic studies revealed swollen endothelial cells and gaps between the cells in regions of intravascular fibrin deposition and a direct interaction of the fibrin fibers with the endothelial cell membrane. These observations, however, were made in animals with experimentally induced disseminated intravascular coagulation and thus, the cause of the findings remains uncertain. Some indication of a direct effect of deposition of fibrin on the vascular endothelium was obtained from experiments with cultured bovine aortic endothelial cells. Contact of a confluent monolayer of those endothelial cells with a fibrin clot resulted in disorganization of the normal monolayer architecture and exposure of subendothelium. In contrast, gels of methylcellulose and agarose had no effect on the morphology of the cells, indicating that mechanical pressure and impaired diffusion of metabolites and oxygen could not explain the phenomenon. Since the underlying mechanism of the fibrin-induced disorganization of endothelial cell monolayers was obscure, the present study was designed to characterize the structural components of the fibrin clot that are required to induce disorganization.
Methods

Reagents

Cell culture media and fetal calf serum (FCS) were obtained from Gibco Europe (Karlsruhe, West Germany); the tissue culture plastic ware was from Becton-Dickinson (Heidelberg, West Germany). Human serum albumin, insulin, actinomycin D, cycloheximide, iodoacetamide, neuraminidase (Type X), and agaroase (Type I, gel point 36°C) were purchased from Sigma (München, West Germany), bovine thrombin (333 NIH units/mg), fibrinogen, fluorescent-labeled rabbit antibodies to human factor VIII:Ag, rabbit anti serum to human fibronectin, to human fibrinogen fragment D and E, respectively from Behringwerke (Marburg, West Germany). Type I collagen (Vitrogen 100) was provided by Flow laboratories (McLean, Virginia) and 3H-proline (Specific activity: 4.8Ci/mmol) by New England Nuclear (Drerich, West Germany). Glycyl-prolyl-arginyl-proline (Gly-Pro-Arg-Pro), gelatin, and lysine were obtained from Serva (Heidelberg, West Germany) and heparin (Liquemin) from Hoffmann La Roche (Basel, Switzerland). Aprotinin (Trasylol) was obtained from Bayer (Leverkusen, West Germany), Hirudin (1000 ATU/mg) and Reptilase (Bathroboxin; 500 U/mg) from Pentapharm (Basel, Switzerland). Sepharose 4 B, DEAE-Sephacel, and QAE-Sephadex were purchased from Pharmacia (Freiburg, West Germany) and polyethylene-terephthalato polyester films (GelBond) from FMC Corporation (Rockland, Maryland).

Endothelial Cell Culture

Human endothelial cells were isolated from umbilical veins as described by Jaffe.16 The resuspended cells were plated in multwell plates of 16 mm diameter that had been precoated with fibronectin and were grown to confluence in Waymouth MB 752/1 medium supplemented with 30% FCS, 10 μg/ml insulin, 20 U/ml heparin, 200 U/ml penicillin, 200 μg/ml streptomycin, and 2 mM glutamine. Cells were identified by their typical cobblestone appearance and stained positively for factor VIII:Ag by direct immunofluorescence. Experiments were exclusively performed with primary cultures. FCS was replaced in all experiments by serum-free medium (SFM) containing 0.1% human serum albumin.

Measurement of Protein Synthesis

De novo protein synthesis was estimated by measuring 3H-proline incorporation into trichloroacetic acid (TCA) precipitable material.17

Purification of Proteins

Fibrinogen

Fibrinogen was isolated from human plasma by the glycine precipitation procedure as previously described in detail.19 Further purification steps included affinity chromatography on lysine-Sepharose and gelatin-Sepharose to remove plasminogen and fibronectin, respectively.20,21 The fibrinogen obtained contained more than 95% crystalline protein and was free of plasminogen and fibronectin as judged by double-immunodiffusion.

Fragment D-cate

Fragment D-cate was purified from human fibrinogen (Kabi, Stockholm, Sweden). After exhaustive dialysis against Tris (20 mM) buffered saline (pH 7.4) in the presence of hirudin (1 ATU/ml), fibrinogen (200 mg) was incubated with plasmin (5 casein units, Kabi) for 16 hours in the presence of 2.5 mM CaCl2.22 The reaction was terminated by addition of aprotinin (1000 KIU/ml). The resulting digest was passed over lysine-Sepharose to remove plasmin and was subjected to chromatography on QAE-Sephadex to separate the fragments.23 Purity was assayed by polyacrylamide gel electrophoresis in the presence of SDS and by double-immuno-diffusion against antisera specific for fragment D and E, respectively. In addition, purified fragment D-cate was functionally tested in a clotting assay for its ability to inhibit polymerization of fibrin.25

Antibodies against Human Fibrinogen

The antibodies were raised in goats and the IgG fraction from the resulting antiserum was prepared by sodium sulphate precipitation of the serum, followed by ion exchange chromatography of the dissolved precipitate on DEAE-Sephacel.

Human Thrombin

Human thrombin (specific activity: 2200 NIH units/mg) was kindly provided by Dr. Klaus T. Pressnner.

Modification of Fibrinogen or Fibrin Clots

Glycosylated Side Chains

Glycosylated side chains were modified by incubation of fibrinogen with 0.05 U neuraminidase for 24 hours before an asialo-fibrin clot was formed.26

Noncrosslinked Fibrin

Noncrosslinked fibrin was prepared by clotting fibrinogen in the presence of 200 mM iodoacetamide, which irreversibly blocks factor XIIIa by carboxymethylation of its sulphhydryl groups.27

Denatured Fibrin Clots

Denatured fibrin clots were prepared as follows: a solution of fibrinogen dissolved in SFM (1 mg/ml) was filled in empty multwell plates of 16 mm diameter (500 μl/well). Bovine thrombin (1 NIH unit/ml) was added and the solution was allowed to clot overnight. Clots were incubated with solutions of glutaraldehyde (6.25%), formaldehyde (4%), or absolute ethanol for 24 hours. Thereafter, the clots were washed with 1 M lysine to bind residual glutaraldehyde and formaldehyde. After extensive washes with SFM, the clots were transferred onto endothelial cells. In another approach, clots were boiled for 15 minutes before they were transferred into multifwells with endothelial cells.

Preparation of Soluble Fibrin

Purified fragment D-cate and the tetrapeptide Gly-Pro-Arg-Pro28 were used to keep fibrin in solution. Bovine thrombin (1 NIH unit/ml) was added to mixtures of fibrinogen/D-cate (0.4/2.4 mg/ml) and fibrinogen/Gly-Pro-Arg-Pro (0.8/3.0 mg/ml) and incubated for 16 hours. Then a sample was taken from both mixtures to determine the release of fibrinopeptides A by radioimmunocassay (Amersham, Braunschweig, West Germany). In another approach, fibrin monomers (0.4 mg/ml) were generated from a preformed fibrin clot in 3 M urea and kept in solution by a tenfold excess of fibrinogen.29
Loading of Fibrin Clots with Antibodies

Preformed clots (1 mg/ml) were incubated with 5 ml of an IgG fraction of a goat antihuman fibrinogen antiserum (9 mg/ml) for 2 hours.

Preparation of Collagen and Agarose Gels

Collagen gels were prepared from acid-dissolved bovine type I collagen (Vitrogen 100) as described previously.\textsuperscript{17} Agarose (2%) containing SFM was kept soluble by maintaining the temperature at 40°C. This solution was mixed 1:1 (vol/vol) with either fibrinogen (2 mg/ml) or human serum albumin (2 mg/ml), both dissolved in SFM. The resulting mixtures were pipetted into multiwells with endothelial cells and allowed to gel. Agarose/fibrin gels were prepared by the addition of bovine thrombin (1 NIH unit/ml) to agarose/fibrinogen mixtures after gelling had occurred.

Preparation of Protein-coated Polyester Films

Hydrophobic polyester films of polyethyleneterephthalate (Gel Bond) that are rendered hydrophilic on one side by coverage with a thin layer of dried agarose, were cut into small pieces of approximately 0.3 × 0.3 mm. The films were incubated for 4 hours in SFM containing either 1 mg/ml fibrinogen, 1 mg/ml fibronectin, 10 mg/ml gelatin, or 10 mg/ml albumin.

Fibrin-coated films were prepared by incubating fibrinogen-coated films with a solution of bovine thrombin (5 NIH units/ml) for 1 hour. Either preincubated films or untreated films were placed on endothelial cells with the hydrophobic or the hydrophilic surface in contact with the cell layers.

Results

Characterization of Disorganization of Endothelial Cell Monolayers

When a solution of fibrinogen was converted to fibrin on top of a monolayer of human endothelial cells, the cells separated in part from adjacent cells and uncovered intercellular spaces, which enlarged with time. The final appearance after 24 hours was a network of tightly packed endothelial cell strands and clusters separated by large cell-free areas (Figure 1 A and B). This effect of fibrin was observed with every endothelial cell strain tested (n = 63).

Disorganization of the monolayers was reversible when the fibrin clot was removed from the cells after 4 hours or was lysed with plasmin. A confluent monolayer displaying the typical cobblestone appearance was reestablished. With prolonged incubation time, the cells became increasingly attached to the fibrin clot and after 24 hours virtually all the cells remained attached to the clot when it was removed. Furthermore, when this clot with the attached...
cells was turned upside down, the cells reorganized on top of the clot from the disorganized pattern to a normal monolayer within 4 hours.

Fibrin-induced disorganization of the monolayer occurred in the presence of actinomycin D (0.1 μg/ml) or cycloheximide (10 μg/ml), i.e., the response of the cells did not require de novo protein synthesis. The effectiveness of the drugs was proven by the reduction of 3H-proline incorporation into TCA precipitable material; the residual protein synthesis of the cells was 20% with actinomycin D and 7% with cycloheximide. In contrast, disorganization was abolished when the experiment was performed in the cold at 4°C.

Effect of Thrombin

Human thrombin led to alterations of human endothelial cell monolayers independent of fibrin. At 15 to 30 minutes after exposure to human thrombin (0.1 NIH unit/ml), gaps occurred between the cells. The normal polygonal shape disappeared and the cells displayed a contracted appearance, thereby becoming isolated from adjacent cells. Despite the presence of thrombin, this phenomenon was transient, and 4 hours later a normal monolayer was restored. Aggregation of the cells to clusters did not occur in the presence of human thrombin. A possible influence of thrombin on the fibrin-induced changes was excluded by quenching any residual thrombin activity with hirudin (10 ATU/ml) after the solutions of fibrinogen were allowed to clot for 4 hours.

Effects of Modified Fibrin Clots

Fibrin clots prepared from purified fibrinogen free of plasminogen and fibronecin induced the same morphological changes as clots from fibrinogen preparations of lower purity. When reptilase was used instead of bovine thrombin to prepare a clot, a fibrin clot devoid of the fibrinopeptides A (desAA-fibrin) formed, which induced the same changes as desAABB-fibrin in which the fibrinopeptides A and B had been cleaved off by thrombin. Experiments were also performed to determine that the fibrinopeptides were not responsible for the disorganization of the monolayers. For this purpose, a fibrin clot was prepared in the presence of apronin (100 KIU/ml) to prevent any further proteolysis. After isolation of the clot with a glass rod, the remaining fluid containing the released fibrinopeptides was incubated with the cells. The morphology of the endothelial cells remained unchanged.

The removal of sialic acids from the fibrinogen molecule, which should affect the charge of the molecule, did not impair the ability of fibrin to induce the morphological changes. The use of noncrosslinked fibrin revealed no differences compared to a crosslinked fibrin clot. In addition, when clots were loaded with antibodies against fibrinogen that should mask a large number of epitopes within the molecule, disorganization of endothelial cell monolayers was not influenced. Even harsh treatment of the fibrin clot leading to substantial structural alterations of the clot did not change the typical picture of disorganization in endothelial cell monolayers. For instance, incubation of the clots with denaturing agents, such as glutaraldehyde and formalin, the use of ethanol, and even boiling for 15 minutes before incubation with endothelial cells could not prevent fibrin-induced disorganization of the cell layers.

Effects of Soluble Fibrin and Fibrin Degradation Products

In contrast to those experiments with fibrin clots, soluble fibrin did not cause any alteration of the endothelial cell monolayer although fibrin had been formed as shown by the determination of fibrinopeptide A release. The values obtained radioimmunologically corresponded to a complete conversion of fibrinogen to fibrin. The findings were independent of the inhibitors of fibrin polymerization used in these experiments. The inhibitors by themselves (fragment D-cate, Gly-Pro-Arg-Pro, or fibrinogen) did not block the induction of disorganization by fibrin clots. Fragment D-cate alone, which was free of fragment E as shown by double-immunodiffusion and which displayed an apparent molecular weight of 94,000 daltons in SDS-PAGE, had no microscopically detectable influence on the monolayer morphology. These findings held true when amounts of more than 2.0 mg fragment D-cate were exposed to the endothelial cells. The plasmin digest derived from a fibrin clot containing the high molecular as well as the low molecular weight fibrin degradation products did not alter the endothelial cell monolayer morphology when the plasmin activity was blocked by the addition of apronin (1000 KIU/ml). Disorganization of the monolayers induced by fibrin clots could not be prevented by this mixture. No attempts were undertaken to select for fibrin degradation products with a defined molecular weight with the exception of fragment D-cate. Table 1 summarizes the results obtained.

Effects of Collagen, Agarose, and Glass

Morphological changes similar to those induced by fibrin could be induced by gels of bovine type I collagen, whereas denatured collagen (gelatin) had no detectable effect on the cells. Disorganization of the monolayers did not occur when the cells were covered with an agarose gel or a mixture of agarose and fibrinogen, but took place when a solution of bovine thrombin was poured over an agarose fibrinogen gel on top of the endothelial cells. Incubation of the cells with small pieces of glass coverslips led to morphological changes of the cells indistinguishable from fibrin-induced disorganization (Figure 2).

Similarly, as observed with the fibrin clots, the cells at-

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Table 1. Effects of Modified Fibrin Clots, Soluble Fibrin, and Fibrin Degradation Products

<table>
<thead>
<tr>
<th>Source of fibrin</th>
<th>Disorganization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncrosslinked fibrin</td>
<td>+</td>
</tr>
<tr>
<td>Asialo-fibrin</td>
<td>+</td>
</tr>
<tr>
<td>Preformed clot</td>
<td>+</td>
</tr>
<tr>
<td>Denatured with glutaraldehyde</td>
<td>+</td>
</tr>
<tr>
<td>Denatured with formaldehyde</td>
<td>+</td>
</tr>
<tr>
<td>Denatured by 15 min boiling</td>
<td>+</td>
</tr>
<tr>
<td>Dehydrated with ethanol</td>
<td>+</td>
</tr>
<tr>
<td>Loaded with antibodies against fibrinogen</td>
<td>+</td>
</tr>
<tr>
<td>Soluble fibrin kept in solution by fibrinogen</td>
<td>0</td>
</tr>
<tr>
<td>Fragment D-cate</td>
<td>0</td>
</tr>
<tr>
<td>Gly-Pro-Arg-Pro</td>
<td>0</td>
</tr>
<tr>
<td>Fibrinopeptides</td>
<td>0</td>
</tr>
<tr>
<td>Unfractionated fibrin degradation products</td>
<td>0</td>
</tr>
<tr>
<td>Fibrinogen fragment D-cate</td>
<td>0</td>
</tr>
</tbody>
</table>

Data represent the response of the cells after 24 hours of exposure to the substances to be tested. Four independent experiments were performed, each experiment in quadruplicate (n = 16). + = disorganization of monolayers; 0 = no disorganization.
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**Table 2. Effects of Gels and Solid Material**

<table>
<thead>
<tr>
<th>Material</th>
<th>Disorganization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>0</td>
</tr>
<tr>
<td>Agarose + fibrinogen</td>
<td>0</td>
</tr>
<tr>
<td>Agarose + fibrinogen + thrombin</td>
<td>+</td>
</tr>
<tr>
<td>Collagen type I, gel</td>
<td>0</td>
</tr>
<tr>
<td>Collagen type I, denatured</td>
<td>0</td>
</tr>
<tr>
<td>Glass</td>
<td>+</td>
</tr>
</tbody>
</table>

Data represent the uniform response of the cells in three series of experiments when judged after 24 hours. Each experiment was carried out four times (n = 12). + = disorganization; 0 = no disorganization.

**Table 3. Effects of Coated Polyester Films**

<table>
<thead>
<tr>
<th>Characterization of exposed material</th>
<th>Disorganization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophilic surface</td>
<td>0</td>
</tr>
<tr>
<td>Preincubated with fibrinogen</td>
<td>0</td>
</tr>
<tr>
<td>Preincubated with fibrinogen + thrombin</td>
<td>+</td>
</tr>
<tr>
<td>Preincubated with fibronectin</td>
<td>0</td>
</tr>
<tr>
<td>Preincubated with gelatin</td>
<td>0</td>
</tr>
<tr>
<td>Preincubated with albumin</td>
<td>0</td>
</tr>
<tr>
<td>Hydrophobic surface</td>
<td>(+)</td>
</tr>
<tr>
<td>Preincubated with fibrinogen</td>
<td>+</td>
</tr>
<tr>
<td>Preincubated with fibrinogen + thrombin</td>
<td>+</td>
</tr>
<tr>
<td>Preincubated with fibronectin</td>
<td>+</td>
</tr>
<tr>
<td>Preincubated with gelatin</td>
<td>+</td>
</tr>
<tr>
<td>Preincubated with albumin</td>
<td>(+)</td>
</tr>
</tbody>
</table>

Data represent the response of the cells in four series of experiments, each performed four times (N = 16). + = disorganization; 0 = no disorganization; (+) = disorganization but at the margins of the films only.

**Effects of Polyester Films on the Integrity of the Monolayers**

The effect of different surface properties exerting the same mechanical pressure on top of the cells were studied by using hydrophobic plastic films of polyethylene terephthalate, which are rendered hydrophilic on one side. When the hydrophobic surface was in contact with the cells, morphological changes occurred but only in small areas of the plastic pieces around the cut edges. These changes induced by the hydrophobic surface could be markedly enhanced when the films were preincubated with solutions containing fibrinogen (1 mg/ml), fibronectin (1 mg/ml), or gelatin (10 mg/ml); i.e., disorganization was found under the whole area of the film and was morphologically identical to the changes induced by fibrin gels and glass. Preincubation with albumin (10 mg/ml) showed no difference compared to the untreated hydrophobic side alone.

In contrast to the hydrophobic surface, the hydrophilic side did not change the morphologic appearance of the cells even after preincubation with fibrinogen, fibronectin, and gelatin. When, however, fibrinogen-covered films were incubated with a solution of bovine thrombin before they were transferred to the top of the endothelial cell surface, disorganization occurred. The cells always attached to the films when disorganization was induced as already mentioned for fibrin clots and glass, whereas the cells did not attach to the hydrophilic surface, i.e., the plastic pieces were floating in the culture dishes when they were slightly agitated. No appreciable variation was observed from one experiment to another. The results are summarized in Table 3.

**Effect of Antibodies against Fibronectin on Endothelial Cells**

The possible role of fibronectin in the mediation of disorganization of the monolayers was addressed in the following experiments. To block any fibronectin present on the luminal surface of the endothelial cells, a monospecific antiserum against human fibronectin was incubated with the endothelial cells, and in experiments performed in parallel, normal rabbit serum was used. Incubation of the antiserum with the cells resulted in pronounced cell detachment from the plastic dishes, whereas controls remained unaffected. Dilutions of the antiserum abolished detachment of the cells and allowed the subsequent exposure of fibrin clots and pieces of glass on top of the cell layers. Under these conditions, no blocking effect on the disorganization process of the monolayers was observed.
Discussion
Deposition of polymerizing fibrin on the vascular endothelium is the final event in intravascular coagulation which might subsequently lead to an occluding thrombus. Fibrin clots exposed to confluent monolayers of human umbilical vein endothelial cells induce disorganization of the monolayer. This disorganization is different from the morphological alterations of the cells observed in response to thrombin. Although the thrombin-induced changes may be important as an immediate reaction of the endothelium in vivo, they are clearly different from the fibrin-induced disorganization process. The fibrinopeptides, which are released during the clotting process, also did not account for the morphological changes described here. The recently observed effect of the fibrinopeptide B_{14}^{14} on the monolayer architecture of the endothelial cells was not detected, since the peptide B_{14}^{14} could not form in the presence of the protease inhibitor, aprotinin. The disorganization of the cells that is seen after exposure to a fibrin clot also seems to be different from those described for fibrin degradation products. Studies with fibrin degradation products, especially those of low molecular weight, have shown an increased permeability of the vessel wall in vivo and an effect on function and morphology of cultured endothelial cells.

The present study did not reveal any influence of an unfractionated lysate of a fibrin clot on the monolayer architecture when the plasmin activity was quenched with aprotinin. In particular, the recent findings by Dang and coworkers that the fibrinogen fragment, D-cate, has a morphologically detectable deleterious effect on human endothelial cells resulting in detachment of the cells from the culture dish could not be confirmed, although even higher concentrations of the fragment D-cate were used.

It is important to stress that every preparation of polymerized fibrin, even denatured clots and clots covered with large amounts of fibrinogen antibodies, induced disorganization, whereas soluble fibrin did not cause any detectable changes in the morphology of the cells. Thus, the structural characteristics of high molecular weight polymers of soluble fibrin that have been demonstrated by gel filtration studies cannot be responsible for the induction of monolayer disorganization. Altogether, the data suggest that the induction of disorganization is not due to a defined structural component of the fibrin molecule. The finding that pieces of glass could alter the morphology of cell monolayers in a way that was indistinguishable from the disorganization induced by fibrin clots, together with the observation that disorganization was always accompanied by cell attachment to the overlying material, prompted us to develop a model that allowed testing of different surface charges and the exposure of those proteins that do not form gels. These experiments, which used polyester films of polyethylene terephthalate, demonstrated that adsorption of those proteins to the hydrophobic surface of the films, which amplify cell attachment in vitro, could amplify disorganization. As already proposed by Kadish and colleagues, fibrotenet that mediates attachment of cells and binds to collagen and fibrinogen may be involved in the attachment of the endothelial cells to the new surface. While energy is required for the disorganization process of the endothelial monolayers as shown by its inhibition in the cold, de novo protein synthesis is obviously not necessary. Thus, a new expression of fibronectin or of another cellular component that might mediate attachment on the former luminal part of the endothelial cell membrane during the disorganization process could not be proven by the use of inhibitors of protein synthesis or by the use of antibodies against fibronectin, since detachment of the cells from the plastic dish occurred.

Although the experiments indicate that induction of disorganization of endothelial cell monolayers is not the result of a specific interaction between the fibrin molecule and the endothelial cell, fibrin could lead to the described alterations under in vivo conditions. Endothelial cell disorganization in vivo may represent a late event after thrombus formation, most likely preceded by the effects of thrombin and the B_{14}^{14} peptide that is cleaved off during early fibrinolysis. These early effects may account for permeability changes of the vessel wall and may be responsible for the accompanying edema in the acute phase of thrombosis, while, on the other hand, the fibrin-induced disorganization of the vascular endothelium could be important for the initiation of a neovascular response during thrombus organization. Initiation of endothelial cell growth was observed when collagen gels induced disorganization of the monolayers, but no growth-promoting effect for fibrin was detected. Additional support for the concept that disorganization of endothelial cells is the initial step required for the recanalization of an occluding thrombus was provided by the recent finding that lumina form and new vessels develop when capillary endothelial cells were covered with collagen gels.

Acknowledgments
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