# Fibrin II Induces Endothelial Cell Capillary Tube Formation

Diana G. Chalupowicz, Zinnat A. Chowdhury, Tami L. Bach, Carl Barsigian, and Jose Martinez

Cardeza Foundation for Hematologic Research and Division of Hematology, Department of Medicine, Jefferson Medical College of Thomas Jefferson University, Philadelphia, Pennsylvania 19107

*Abstract.* We studied the formation of capillary tubes by endothelial cells which were sandwiched between two fibrin gels under serum-free conditions. After formation of the overlying fibrin gel, the endothelial cell monolayer rearranged into an extensive net of capillary tubes. Tube formation was apparent at 5 h and was fully developed by 24 h. The capillary tubes were vacuolated, and both intracellular and intercellular lumina were present. Maximal tube formation was observed with fibrin II (which lacks both fibrinopeptide A and B), minimal tube formation with fibrin I (which lacks only fibrinopeptide A), and complete absence of tube formation with fibrin 325 (which lacks the $NH_2$-terminal $\beta15-42$ sequence, in addition to fibrinopeptides A and B). The inability of fibrin 325 to stimulate capillary tube formation supports the idea that $\beta15-42$ plays an important role in this process, and its importance was confirmed by the finding that exogenous soluble $\beta15-42$ inhibited fibrin II-induced capillary tube formation. This effect was specific for fibrin, since $\beta15-42$ did not inhibit tube formation by endothelial cells sandwiched between collagen gels. The interaction of the apical surface of the endothelial cell with the overlying fibrin II gel, as opposed to the underlying fibrin gel upon which the cells were seeded, was necessary for capillary tube formation. These studies suggest that the $\beta15-42$ sequence of fibrin interacts with a component of the apical cell surface and that this interaction plays a fundamental role in the induction of endothelial capillary tube formation.

A NGIOGENESIS, which is the formation of new blood vessels, plays a fundamental role in many physiological and pathological processes including wound healing, tumor growth, and tissue repair following inflammation (2, 12, 15, 17). Because of the broad context in which angiogenesis is involved, a variety of in vitro assays have been developed that attempt to approximate conditions which occur in vivo. These models involve the culture of endothelial cells on purified matrix proteins such as collagen (16, 29, 34, 35), fibrin (18, 30, 34, 35), fibronectin (22, 26), or on complex multimolecular matrices such as Matrigel (20, 23, 35). Under the appropriate conditions, endothelial cells migrate into the protein matrix gel and undergo a shape change, resulting in the formation of a net-like array of capillary-like tubes which may contain lumina (16, 35). Depending on the model used, this process may require cell proliferation and migration into the gel through controlled proteolysis of the matrix proteins (30, 37, 43).

Several specific regulatory factors, such as fibroblast growth factor, platelet-derived growth factor, and vascular endothelial growth factor, seem to be involved in the induction as well as the propagation of angiogenesis (4, 14,

17, 26, 31). The interplay of these angiogenic growth factors with factors supporting the propagation of angiogenesis, most notably specific molecular components of the extracellular matrix (1, 9–11), appears to be crucial for morphogenesis of endothelial cells into capillary-like tubes (16, 21, 27). In many angiogenic assays, capillary tube formation requires several days to weeks to occur, while in other systems using specific matrix proteins and experimental conditions, tube formation can sometimes be observed after 1 to 2 d (3, 18, 22, 26). Thus, the type of experimental model used profoundly affects the regulation of angiogenesis under these in vitro conditions.

Fibrin plays a prominent role in wound healing and tumor growth (2, 12, 15), and has, therefore, frequently been used as a matrix model system designed to test the effect of angiogenesis-inducing compounds (18, 30, 35). In this report, we describe a novel serum-free system where human umbilical vein endothelial cells (HUVEC)[1] are sandwiched between two fibrin gels. When the second fibrin gel is formed overlying the endothelial cell monolayer, the cells rapidly differentiate into capillary tubes. This system allows for analyses of the effects of the overlying versus underlying fibrin gels and also of the molecular structure of fibrin which is active in the induction of capillary tube

Address all correspondence to Jose Martinez, M.D., Cardeza Foundation for Hematologic Research, 1015 Walnut Street, Curtis Building, Room 804, Philadelphia, PA 19107-5099. Tel.: (215) 955-7790. Fax: (215) 923-3836.

1. *Abbreviations used in this paper:* HUVEC, human umbilical vein endothelial cells; RGDS, Arg-Gly-Asp-Ser.

formation. The results indicate that it is the overlying gel which mediates the formation of endothelial cell capillary tubes and that the β15–42 sequence located at the $NH_2$ terminus of the fibrin β chain is responsible for the induction of capillary tube formation.

## Materials and Methods

### Reagents

Endothelial cell basal medium was from Clonetics Corp. (San Diego, CA); basic fibroblast growth factor human recombinant and rat tail collagen (type I) were from Collaborative Biomedical Products (Bedford, MA); and Gibco BRL media supplement general (GMS-G) supplement (100X) containing sodium selenite (0.00067 g/liter), insulin (1.00 g/liter), and transferrin (0.55 g/liter) was from GIBCO BRL (Gaithersburg, MD). Lyophilized human thrombin and Atroxin (Bothrops atrox venom) were from Sigma Chemical Co. (St. Louis, MO). Carrier-free $^{125}I$ (NaI in NaOH) was from Amersham Corp. (Arlington Heights, IL). Reagents for electron microscopy were from Polysciences, Inc. (Warrington, PA). Transparent culture plate inserts (12-mm diameter) were from Millipore Corp. (Bedford, MA). Crotalus atrox protease III was donated by Dr. Andrei Budzynski (Temple University, Philadelphia, PA) and Dr. Perumal Thiargarajan (University of Texas, Health Science Center at Houston, Houston, TX). Monoclonal antibody 7E3 was a gift from Dr. Barry Coller (Mt. Sinai Medical Center, New York). Purified human von Willebrand factor was a gift from Dr. Luigi De Marco (Centro Transfusionale C. R. O., Aviano, Pordenone, Italy). Human arterial smooth muscle cells were donated by Dr. Kerri Pratt (Jefferson Medical College of Thomas Jefferson University, Philadelphia, PA). All other reagents were purchased from Fisher Scientific Co. (Pittsburgh, PA), Sigma Chemical Co., and GIBCO BRL.

### Endothelial Cells

Primary cultures of HUVEC were isolated from umbilical cords and maintained in culture as described (28). HUVEC (passages 1–4) were detached by incubation for 5 min in 0.05% trypsin/0.53 mM EDTA at room temperature. The cells were centrifuged at 60 g for 5 min and resuspended in serum-free endothelial cell basal medium containing bovine serum albumin (1% wt/vol), basic fibroblast growth factor (2–5 ng/ml), insulin (0.01 mg/ml), transferrin (5.5 μg/ml), and sodium selenite (6.7 ng/ml).

### Synthetic Peptides

Peptide β15–42 was synthesized by the Fmoc strategy of solid-phase synthesis using a peptide synthesizer (430A; Applied Biosystems, Inc., Foster City, CA) and purified by an Applied Biosystems semipreparative system equipped with a reverse-phase column (C18; Rainin Instrument Co. Inc., Woburn, MA). Synthesis of the peptide was carried out at the Jefferson Cancer Institute (Philadelphia, PA). The GHRP peptide (β15–18) from the $NH_2$ terminus of the fibrin β chain was purchased from Sigma Chemical Co.

### Capillary Tube Formation by Endothelial Cells Sandwiched between Fibrin Gels

Fibrinogen was purified from human plasma as previously described (28). For some experiments, the purified fibrinogen was chromatographed on DEAE-Sepharose (Sigma Chemical Co.) (33) to remove contaminating plasminogen, von Willebrand factor, fibronectin, and factor XIII. The purified fibrinogen was free of contaminating von Willebrand factor and fibronectin, as demonstrated by Western blot analysis, and of factor XIII, as demonstrated by the absence of cross-linking of the γ chain following clot formation induced by thrombin.

*Preparation of Fibrin II Gels.* Fibrinogen (1–3 mg/ml) was dialyzed into 0.05 M Tris/0.15 M NaCl. To make the underlying fibrin II gel, 225 μl of fibrinogen solution was placed into each well of a 24-well culture plate, and human thrombin (10–30 U/ml in 10× minimum essential medium) was added to a final concentration of 1 U of thrombin/mg of fibrinogen. After the gels were polymerized (at least 5 min at 37°C), 1-ml aliquots of endothelial cells (250,000 cells/ml) suspended in serum-free endothelial cell basal medium, supplemented as indicated above, were seeded onto each fibrin II gel. After 24 h the cells had spread to form a confluent monolayer. After aspiration of the culture medium, the same procedure was

used to generate a second fibrin II gel overlying the apical surface of the cells. This fibrin II gel was allowed to polymerize for ~5 min at 37°C, and then 1-ml aliquots of fresh, supplemented, serum-free endothelial cell basal medium were added to each well. In some experiments, overlying preformed fibrin or collagen gels, prepared on the reverse side of transparent culture plate inserts (Millipore Corp.), were gently placed, gel side down, on the surface of the cell monolayers. Fresh media were then added to the interior of the plastic inserts, as well as to the wells into which the inserts had been placed.

*Preparation of Fibrin I Gels.* Fibrin I gels were prepared in the same manner as fibrin II gels except that Atroxin (10–30 μg/ml in sterile distilled water) was added in place of thrombin to a final concentration of 1 μg/mg of fibrinogen. In experiments where fibrin I was generated on top of monolayers established on fibrin II made with thrombin, residual thrombin was neutralized with hirudin (1 U/ml) for 30 min at 37°C.

*Preparation of Fibrin-325 Gels.* Fibrinogen 325, which lacks the Bβ1–42 amino-terminal sequence, was prepared by digestion of fibrinogen with protease III from Crotalus atrox at an enzyme-to-fibrinogen ratio of 1:1,000 (36). The digestion was carried out in 50 mM ammonium bicarbonate (pH 8.0) containing 2 mM EDTA for 3 h at 37°C. The action of the protease was terminated by addition of phenylmethylsulfonyl fluoride to a concentration of 1 mM. The fibrinogen 325 was dialyzed extensively against 50 mM Tris (pH 7.5) and stored at −20°C. Underlying and overlying fibrin gels were prepared as described above for fibrin II with the following exceptions. Fibrinogen 325 was in 50 mM Tris (pH 7.5) to which were added calcium chloride (final concentration 1 mM) and human factor XIII (final concentration 10 μg/ml). Thrombin (dissolved in 50 mM Tris) was added to a final concentration of 10 U/ml, and the fibrin was allowed to gel for 1–2 h. Control fibrin II and fibrin I gels for these experiments were prepared in 50 mM Tris with added calcium chloride and factor XIII and either thrombin (10 U/ml) or Atroxin (1 μg/ml), respectively.

### Capillary Tube Formation by Endothelial Cells Sandwiched between Collagen Gels

Type I collagen from rat tail was diluted to a concentration of 1 mg/ml, and the pH was neutralized by adding one-tenth of the volume of 10 × MEM. 250-μl aliquots were immediately added to each well of 24-well culture plates and incubated at 37°C until gelation occurred. Endothelial cells were seeded on the collagen-coated wells and incubated for 24 h. After aspiration of unattached cells, overlying collagen gels were generated. In some experiments, preformed collagen gels were placed on top of the monolayers as described above for fibrin II.

### Inhibition of Capillary Tube Formation by Peptides or Antibodies

To study inhibition by the peptides β15–42 or β15–18, endothelial cells were seeded on either fibrin II gels prepared from fibrinogen (0.5 mg/ml) or on collagen gels (1 mg/ml) prepared as described above. After 24 h the media were replaced by fresh media containing 1 mM β15–42 or β15–18. After 1 h of incubation, the media were removed, and preformed fibrin or collagen gels on plastic culture plate inserts were gently placed on top of the cells. Fresh media containing the same concentration of peptides was added to the interior of the plastic inserts, as well as to the wells into which the inserts had been placed. Controls were treated similarly, but no peptide was present in the culture media. To study inhibition by Arg-Gly-Asp-Ser (RGDS) or 7E3, endothelial cells were seeded on fibrin II gels as indicated above. After 24 h the media were replaced by fresh media containing the appropriate concentrations of RGDS or 7E3 and incubated for at least 30 min. Media were then removed and fibrin II gels, prepared from fibrinogen solutions containing RGDS or 7E3, were generated on top of the cells. Fresh media containing the agents were then added to the wells.

### Microscopic Analysis of Capillary Tubes

Endothelial cell monolayers and capillary tubes were assessed by phase contrast microscopy. Capillary tubes were defined as straight cellular extensions joining two cell masses or branch points. The measurement of the number, length, and width of capillary tubes was done by digitalized computer microscopy (Power Macintosh 7100/66 computer and Nikon Microphot SA microscope with Sony CCD video camera, using NIH Image, Version 1.55, software) of cells that had been fixed and stained with Leukostate™ Stainkit (Fisher Scientific). In some experiments, photographs were taken of 0.67-mm$^2$ fields which manifested high visual clarity (18).

From the photographs, the number of capillary tubes was determined, and the length and width of the tubes were measured using a calibrated ruler. Statistical analysis was done using the Students $t$ test. For electron microscopic analysis, wells were washed with PBS, and the endothelial cell-fibrin sandwiches containing reorganized capillary tubes were fixed for 24 h at 4°C in a 2.5% glutaraldehyde/2% paraformaldehyde mixture in 0.1 M sodium cacodylate buffer (pH 7.4). The fixed gels were washed with 0.1 M sodium cacodylate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature. The samples were dehydrated in a graded series of acetone and embedded in Spurr's low-viscosity embedding media. Thin sections were cut with a diamond knife and counterstained with uranyl acetate and lead citrate. The stained sections were examined, and photomicrographs were obtained using a transmission electron microscope (7000; Hitachi Ltd., Tokyo, Japan).

## SDS-Polyacrylamide Gel Electrophoresis

Fibrinogen was solubilized, under reducing conditions, and run using a 4% polyacrylamide stacking gel and a 10% resolving gel (28). The proteins in the gel were visualized by staining with Coomassie brilliant blue. Some gels were transferred to nitrocellulose paper, and Western blots were performed using polyclonal antisera against plasminogen, von Willebrand factor, or fibronectin. Protein bands were visualized using secondary antibody conjugated with horseradish peroxidase. The polypeptide chain composition of fibrin II, fibrin I, and fibrin 325 was assessed by 7% SDS-PAGE after disulfide bond reduction (41).

# Results

## HUVEC Monolayers Sandwiched between Fibrin II Gels Rapidly Form Capillary Tubes

The rapid formation of capillary tubes in collagen or fibrin gels has been reported to require stimulation of the endothelial cells by the tumor promoter PMA (18). In the present investigation, endothelial cells sandwiched between fibrin II gels formed capillary tube structures within only a few hours, even when cultured in medium devoid of PMA. In these experiments, HUVEC seeded on three-dimensional fibrin II gels established a confluent monolayer within 24 h and remained confluent for up to 2 d in culture (Fig. 1 a). If an overlying fibrin II gel was generated on top of the monolayer, the cells rapidly formed capillary-like tube structures which could be observed within 5 h (Fig. 1 b) and which were usually fully developed into long, thin tubes by 24 h (Fig. 1 c). The process of capillary tube formation by endothelial cells sandwiched between fibrin gels was specific for endothelial cells, since human arterial smooth muscle cells did not rearrange into capillary tubes (Fig. 1 d).
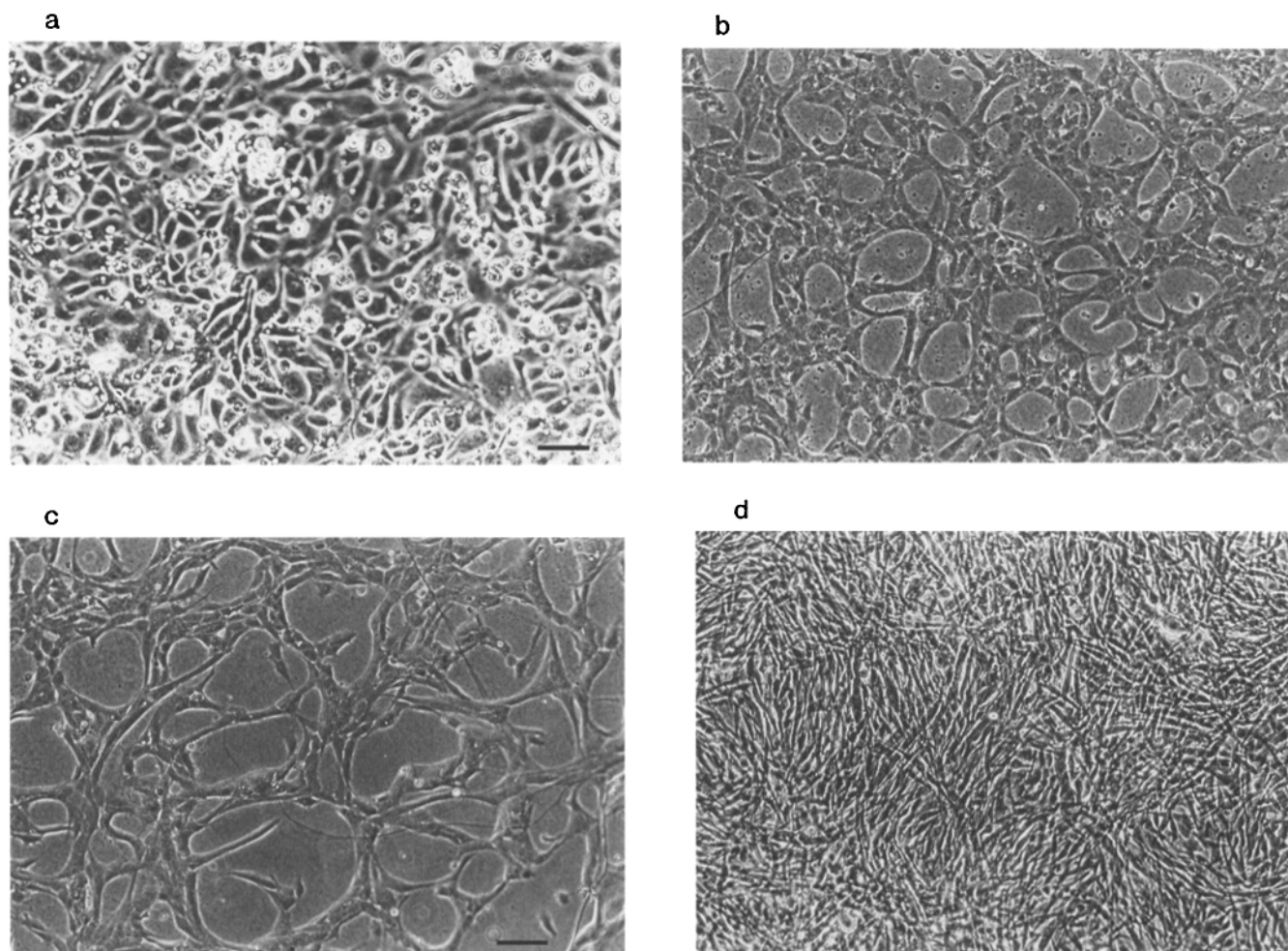


*Figure 1.* Endothelial cells sandwiched between fibrin II gels rapidly form capillary tubes. Cells were grown to confluence on fibrin II gels, and an overlying fibrin II gel was then formed on top of the cell monolayers. Before generation of the overlying fibrin II gel, HUVEC were present as an intact monolayer (a). Within 5 h of generation of the overlying fibrin II gel, the formation of a netlike array of capillary tubes was apparent (b), and by 24 h capillary tube formation was usually fully developed (c). Arterial smooth muscle cells failed to form capillary tubes in response to an overlying fibrin II gel (d). Bars, 120 μm.
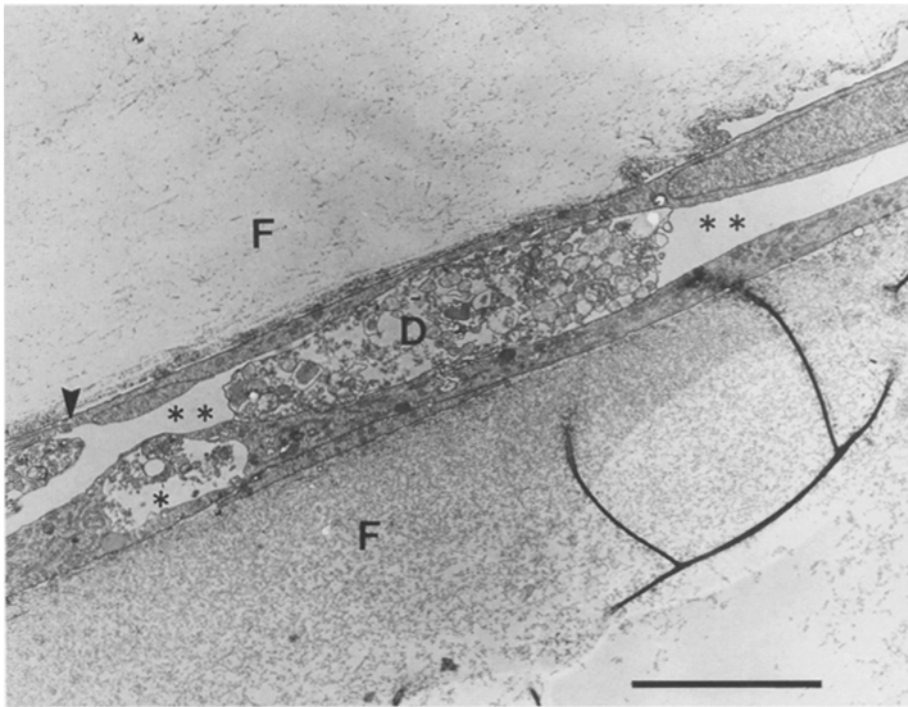
*Figure 2.* Capillary tubes formed between fibrin II gels manifest intercellular and intracellular lumina. Specimens were fixed for 24 h at 4°C in a 2.5% glutaraldehyde/2% paraformaldehyde solution, postfixed in 1% osmium tetroxide, dehydrated in acetone, and embedded in Spurr's low-viscosity embedding media. Thin sections were cut and counterstained with uranyl acetate and lead citrate. In this figure, an endothelial capillary tube has been sectioned along its longitudinal axis to reveal both an intercellular lumen (**) formed by the interdigitation of several endothelial cell processes as well as a putative intracellular lumen (*) present within the cytoplasm of a single cell. Note that the intracellular lumen is lined by a cell membrane. Both lumina are filled with cellular debris (*D*). The intercellular junctions between individual endothelial cells can be seen (*arrows*). The underlying and overlying fibrin gels (*F*) are shown. The underlying fibrin gel appears much denser. ×10,000. Bar, 6 μm.

By light microscopy, the capillary tubes were observed to contain vacuoles and channels and were very similar in overall appearance to endothelial cell capillary tubes observed by other investigators (16, 18). By electron microscopy, debris-filled intercellular lumina, formed by the interdigitation of several endothelial cells, as well as distinct intracellular lumina could be observed (Fig. 2). Proteolysis of the fibrin was not required for tube formation, since aprotinin did not inhibit tube formation and the fibrin gels demonstrated intact α, β, and γ chains in SDS-polyacrylamide gels (not shown). Finally, tube formation was not due to contaminating proteins such as fibronectin, factor XIII, or von Willebrand factor, since depletion of these proteins from fibrinogen was without effect on tube formation (not shown).

Since thrombin itself has been reported to cause endothelial cell retraction (24), experiments were performed to delineate the potential effect of thrombin from that of the fibrin II gel. When thrombin was added to medium overlying an intact endothelial cell monolayer established on a fibrin II gel, rearrangement of the cells was not observed and the cells remained as a monolayer (not shown), indicating that thrombin was not responsible for the formation of capillary tubes induced by fibrin II gels. Furthermore, endothelial cells sandwiched between preformed fibrin II gels that had been previously exposed to hirudin (1 U/ml for 30 min), to neutralize the thrombin, formed normal-appearing capillary tubes (not shown).

### The αvβ3 Integrin Receptor Is Not Essential for the Formation of Capillary Tubes by Sandwiched HUVEC

The αvβ3 integrin, which is the major integrin receptor of endothelial cells currently known to interact with fibrinogen (1, 9–11), has been shown to play a role in regulating

angiogenesis under some experimental conditions (6, 18). We therefore analyzed the role of the αvβ3 integrin receptor under our experimental conditions in which capillary tube formation is induced by sandwiching the endothelial cells between two fibrin gels in a serum-free system that is devoid of phorbol esters. When HUVEC were sandwiched between fibrin II gels and incubated in medium without or with RGDS or the monoclonal antibody 7E3 directed against αvβ3, the formation of capillary tubes was unaltered (Table I). Increasing the concentration of 7E3 to 30 μg/ml had an apparently slight enhancing effect on tube formation similar to that reported by other investigators (18). Overall, our data indicate that disruption of αvβ3 integrin interaction with fibrin did not inhibit capillary tube formation by cells sandwiched between two fibrin II gels.

### HUVEC Monolayers Sandwiched between Fibrin I Gels Fail to Form Capillary Tubes

Fibrin II is formed from fibrinogen by thrombin cleavage of fibrinopeptide A from the Aα chain and fibrinopeptide B from the Bβ chain, resulting in the generation of the α

*Table I. Effects of RGDS or 7E3 on Capillary Tube Formation*

| Treatment | Capillary tube parameters* | | |
| | Number[+] | Length | Width |
| --- | --- | --- | --- |
| | | *μm* | *μm* |
| Control | 26 ± 7 | 122 ± 24 | 25 ± 6 |
| RGDS 150 μm | 25 ± 7 | 117 ± 19 | 26 ± 7 |
| RGDS 500 μm | 23 ± 9 | 102 ± 21 | 22 ± 3 |
| 7E3 10 μg/ml | 27 ± 7 | 124 ± 17 | 25 ± 6 |

*Values are means ± SD of at least 10 experiments for control and 4–7 experiments for treatments.
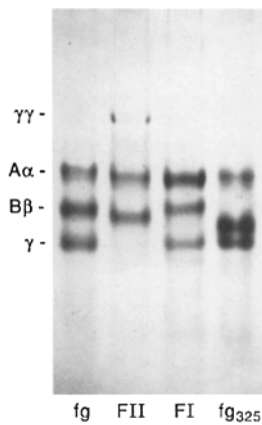[+]Number of tubes in 0.3 mm².

*Figure 3.* Electrophoretic behavior of structurally distinct forms of fibrin. Fibrin II (*FII*) and fibrin (*FI*) were prepared by treatment of fibrinogen (*fg*) with thrombin or Atroxin, respectively. Fibrinogen 325 was prepared by treatment of fibrinogen with protease III. Samples were run on a 7% polyacrylamide gel and stained with Coomassie blue. In fibrin II and fibrin I, the α chain migrates faster than the Aα chain of fibrinogen due to cleavage of fibrinopeptide A. In fibrin II, the β chain also migrates faster that the Bβ chain of fibrinogen due to cleavage of fibrinopeptide B. The β chain of fibrin 325 migrates just above the γ chain due to the absence of Bβ1–42.

and β chains of fibrin II, respectively (25, 39). Thus in fibrin II, new NH₂ termini are generated for both the α and β chains, relative to fibrinogen. In contrast, fibrin I can be formed from fibrinogen by Atroxin cleavage of only fibrinopeptide A; thus, the Bβ chain of fibrin I is the same as that in fibrinogen. These biochemical differences, which can be assessed by SDS-polyacrylamide gel electrophoresis (Fig. 3), account for structural differences between polymerized fibrin I and fibrin II (32), and may provide clues as to the structural features of fibrin that are important for capillary tube formation.

As reported by other investigators (7), endothelial cells seeded onto fibrin I gels remained rounded after 2 h of incubation (not shown), but by 24 h a confluent monolayer of endothelial cells was established on fibrin I gels (Fig. 4 *a*), indicating that fibrin I is capable of providing sufficient support for the eventual establishment of an intact cellular monolayer. Cell spreading on fibrin I gels was integrin mediated, since it was markedly inhibited by RGDS (Fig. 4 *b*) or 7E3 (not shown). Whether the cell spreading on fibrin I in the absence of antiintegrins was supported by the re-

lease of von Willebrand factor from the cells or was directly supported by the fibrin I molecule itself is not known. Nevertheless, the spreading of endothelial cells on the fibrin I gels allowed the study of potential capillary tube formation using the fibrin-gel sandwich technique.

Endothelial cell monolayers sandwiched between fibrin I gels manifested a variable range of responses from one experiment to the next. The extremes ranged from no change in the confluent cobblestone appearance of the monolayer (not shown) to the formation of attenuated tubelike structures (Fig. 5) that were very transient. Formation of these quasi tube structures was not a result of von Willebrand factor secreted by the cells, since the addition of purified von Willebrand factor to the fibrinogen used to generate the fibrin I gels did not result in capillary tube formation (not shown). Thus, there must be structural features in fibrin II, not present in fibrin I, which play an important role in the formation of endothelial cell capillary tubes.

Because it was possible that the Atroxin used to generate fibrin I gels inhibited the formation of capillary tubes, control experiments were performed in which fibrin II gels were made using a mixture of thrombin plus Atroxin. These fibrin II gels, which had the same structure as fibrin II gels made with thrombin alone, induced capillary tube formation that was indistinguishable from tubes formed in fibrin II gels in the absence of Atroxin (not shown), indicating that Atroxin was not inhibitory to capillary tube formation.

## The Overlying Fibrin II Gel Induces the Formation of Capillary Tubes

To determine whether the morphogenesis of the endothelial cells into capillary tube structures was triggered by the interaction of the cells with the underlying fibrin gel used for seeding or with the overlying fibrin gel formed on top of the cells, experiments were done using fibrin II as the underlying gel and either fibrin II or fibrin I as the overlying gel. Under these conditions, the interaction of the cells with the overlying gels could be assumed to be responsible
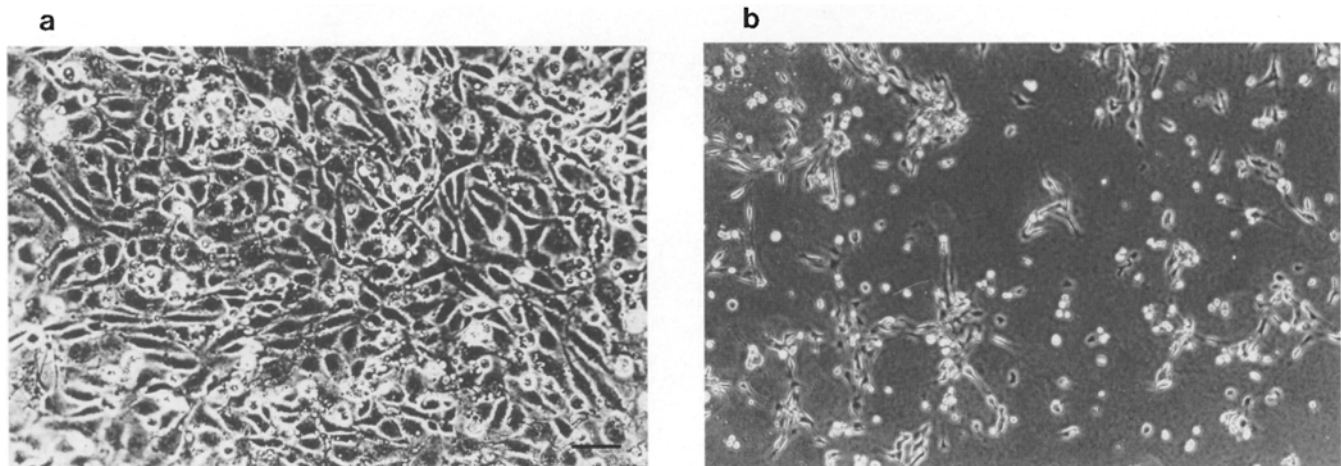


*Figure 4.* Endothelial cell spreading on a fibrin I gel is mediated by cellular integrins. HUVEC were seeded onto a fibrin I gel in the absence (*a*) or presence (*b*) of 500 μm RGDS. After 24 h of culture, control cells established a confluent monolayer, while RGDS completely prevented cell spreading. Bar, 120 μm.
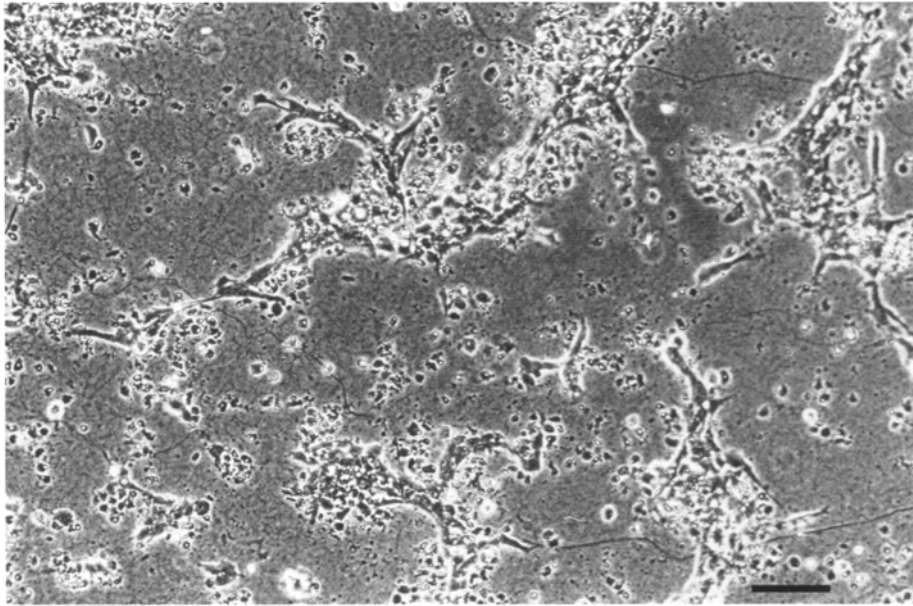
*Figure 5.* Endothelial cells sandwiched between fibrin I gels fail to form capillary tubes. HUVEC were seeded onto fibrin I gels and cultured to confluence. Overlying fibrin I gels were then generated atop the cells and the incubation was continued for an additional 24 h. The HUVEC formed attenuated and transitory semi-tube-like structures. Bar, 120 μm.

for the observed changes in cellular morphology, since cells sandwiched between two fibrin I gels did not form capillary tubes. When fibrin II was generated on top of the cells, well-developed capillary tubes were always seen (see Fig. 1 *c*). In contrast, when fibrin I was generated on top of the cells that had been seeded on fibrin II, a high degree of interexperimental variability was seen. In some experiments, the endothelial cells remained as an intact monolayer (not shown; identical to Fig. 1 *a*), while others yielded capillary tubes that appeared blunted in development (Fig. 6). These tubes were very transient and usually degenerated over the following day in culture. These findings emphasize the importance of the overlying fibrin II gel in the formation of capillary tubes and indicate that structural determinants present in fibrin II, but not in fibrin I, are important for the optimal formation of capillary tubes.

## The β15-42 Sequence in Fibrin II Is Necessary for Endothelial Cell Capillary Tube Formation

The β15–42 sequence mediates the spreading of endothelial cells on fibrin II (7, 13). In order to assess whether this sequence is also important for the morphogenesis of endothelial cells into capillary tubes, experiments were performed using fibrin 325, which is generated from fibrinogen 325 which lacks the amino terminal Bβ1–42 sequence (Fig. 3). In these experiments, capillary tube formation did not occur when endothelial cell monolayers were established on fibrin II, and an overlying fibrin gel was generated from fibrinogen 325. Conversely, when monolayers were established on fibrin 325 and an overlying fibrin II gel was formed, the cells rearranged into capillary tubes within 24 h (not shown). The same result was obtained using preformed fibrin gels that were adherent to plastic in-
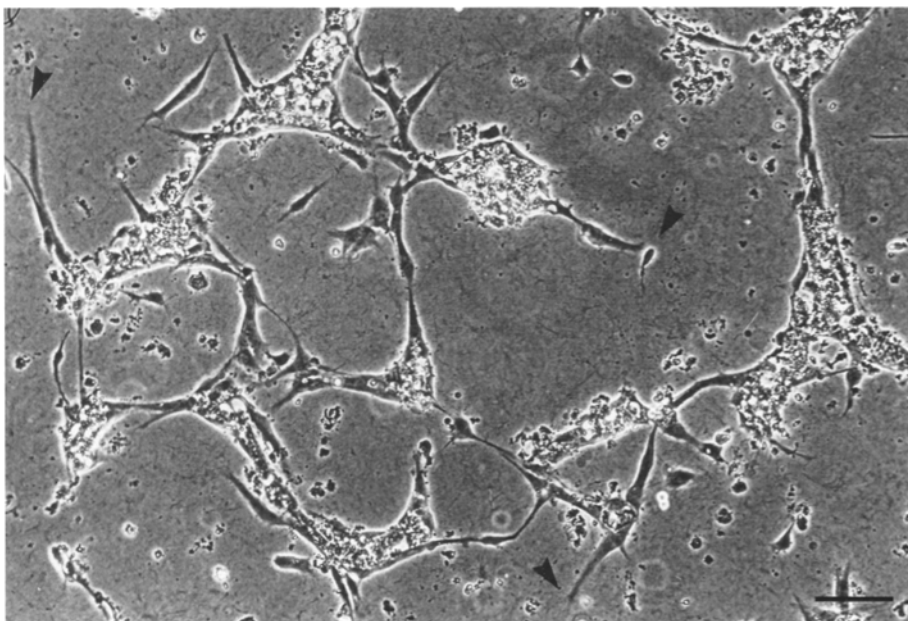


*Figure 6.* Endothelial cells seeded on a fibrin II gel with an overlying fibrin I gel form blunted capillary tubes. HUVEC monolayers were established on fibrin II gels and an overlying fibrin I gel was then generated. The cells formed linear tubelike projections which ended blindly (*arrows*) without establishing many cell–cell contacts and without the resulting netlike appearance of tubes sandwiched between fibrin II gels (see Fig. 1).
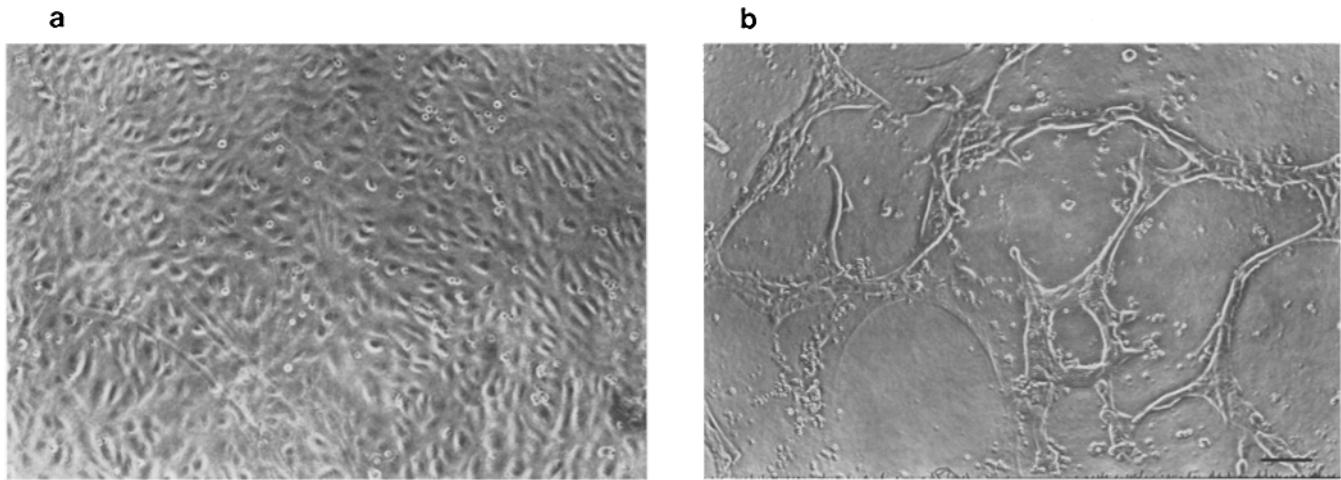
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a

b



*Figure 7.* Interaction of the overlying fibrin II gel with the apical surface of the cell monolayer optimally promotes capillary tube formation. HUVEC monolayers (*a*) were established on fibrin II gels and overlaid with preformed fibrin-325 gels or (*b*) where established on fibrin-325 gels and overlaid with preformed fibrin II gels. With fibrin-325 on top, the cells remained as a monolayer (*a*), and with fibrin II on top the cells formed capillary tubes (*b*). Bar, 120 μm.

serts and then placed on top of the endothelial cell monolayer. When endothelial monolayers were established on fibrin II and a preformed fibrin-325 gel placed on top, the monolayer remained intact (Fig. 7 *a*). In contrast, when monolayers were established on fibrin 325 and a preformed fibrin II gel was placed on top, the cells reorganized into capillary tubes (Fig. 7 *b*).

The importance of the β15–42 sequence within fibrin II in the induction of capillary tube formation was directly demonstrated by the ability of excess soluble β15–42 peptide to inhibit capillary tube formation induced by fibrin II. The addition of 1 mM β15–42 to the culture media before the addition of a prepolymerized overlying fibrin II gel significantly reduced the number and length of the tubes, while significantly increasing tube diameter (Table II). The tetrapeptide GHRP (β15–18) did not induce similar alterations, nor did β15–42 alter capillary tube formation by endothelial cells sandwiched between collagen gels (not shown), indicating the specificity of the fibrin II–endothelial cell interaction.

In addition to establishing the importance of the β15–42 sequence in fibrin II–induced endothelial cell capillary tube formation, the above results also indicate that thrombin was not responsible for tube formation since the preformed fibrin-325 gels contained active thrombin, yet did not induce tube formation. They also corroborate the observation that it is the overlying fibrin II gel interacting with the apical surface of the endothelial cell monolayer

which is responsible for induction of the morphogenesis process.

## Discussion

In this work, we demonstrate that HUVEC, when sandwiched between two fibrin II gels, promptly rearrange into an extensive network of capillary-like tubes. These structures were clearly discernible as early as 5 h after formation of the overlying fibrin II gel and remained intact for up to 3 d. Consistent with the features of capillary tubes studied by others (16, 18), light microscopy revealed the presence of vacuoles and channels within the tubes that, by electron microscopy, were seen to contain both intercellular and intracellular lumina which were partially filled with cellular debris. The formation of these structures was directly supported by the fibrin II molecule itself, since the depletion of contaminating proteins, such as fibronectin, von Willebrand factor, factor XIII, and plasminogen, from the purified fibrinogen used to generate the fibrin II did not hinder capillary tube formation. Moreover, $\alpha_v\beta_3$ interaction with von Willebrand factor, which may have been secreted by the cells in response to fibrin II (38), did not appear to be responsible for tube formation since the addition of exogenous von Willebrand factor to the fibrinogen used to form the fibrin I gels did not promote tube formation.

A unique and important finding of this study is that generation of capillary tubes was optimally supported by a specific form of fibrin, namely, fibrin II. Tube formation was only minimally supported by fibrin I and never observed with fibrin 325. In vivo, fibrin II formation predominates over fibrin I formation, although both forms have been demonstrated in vascular lesions (5). The newly formed fibrin monomers are susceptible to the action of plasmin, which cleaves β15–42 (42) generating fibrin 325, which manifests markedly delayed polymerization (40). Fibrin 325 can also be generated experimentally from fibrinogen 325, a fibrinogen derivative prepared by treatment of fibrinogen with protease III from *Crotalus atrox*,

*Table II. Inhibition of Fibrin II–induced Capillary Tube Formation by Soluble β15–42*

| Treatment | Capillary tube parameters* | | |
|---|---|---|---|
| | Number‡ | Length | Width |
| | | *μm* | *μm* |
| Control | 12.7 ± 4.3 | 101.0 ± 24.4 | 7.6 ± 1.6 |
| β15–42 | 7 ± 1.4§ | 66.7 ± 14.8§ | 12.8 ± 2.4§ |

*Values are means ± SD of at least four experiments.
‡Number of tubes in 0.3 mm².
§Significantly different from control; *P* < 0.05.

which releases the Bβ1–42 sequence from the NH₂ terminus of each fibrinogen Bβ chain thereby reducing the molecular mass to 325 kD (36). In addition to its role in fibrin polymerization, the β15–42 sequence has also been shown to inhibit fibrinogen binding to platelets via a mechanism which may involve its binding to fibrinogen, as opposed to the platelet fibrinogen receptor GPIIb/IIIa (8). It also mediates fibrin II–induced release of von Willebrand factor from endothelial cells (13, 38), and accounts, at least in part, for the ability of fibrin II to support spreading of endothelial cells (7, 13). Our demonstration of the inability of fibrin I or fibrin 325 to trigger endothelial cell capillary tube formation and the ability of soluble β15–42 to inhibit tube formation induced by fibrin II, but not by collagen, indicates the importance of the β15–42 sequence as a structural determinant of fibrin II that may account for this activity.

Our observation that endothelial cells fail to spread on fibrin I within 2 h is in agreement with findings of other investigators (7). However, our demonstration of the capacity of endothelial cells to spread on fibrin I after 24 h allowed us to study the formation of capillary tubes by endothelial cell monolayers sandwiched between fibrin I gels. Our results showing that RGDS and the monoclonal antibody 7E3 disorganize endothelial cell monolayers plated on fibrin I, but not on fibrin II, indicate that the $\alpha_v\beta_3$ integrin plays a major role in maintaining the integrity of endothelial cell monolayers plated on fibrin I, but not on fibrin II. These data further indicate that other determinants within fibrin II, notably the β15–42 sequence, are capable of supporting endothelial cell spreading as previously reported (7, 13).

The high level of expression of $\alpha_v\beta_3$ in granulation tissue indicates that this integrin is involved in angiogenesis (6). Indeed, in the chick chorioallantoic membrane, antibodies against $\alpha_v\beta_3$ have a potent inhibitory effect on the formation of new blood vessels (6). In contrast to this inhibitory effect, PMA-induced formation of capillary tubes on collagen or fibrin gels is enhanced by antibodies to the $\alpha_2\beta_1$ integrin or the $\alpha_v\beta_3$ integrin, respectively (18). Thus, the role of $\alpha_v\beta_3$ in angiogenesis is complex and the observed effect on capillary tube formation may depend, in part, on the experimental conditions. Indeed, in our system, neither RGDS nor the antibody 7E3 against the β₃ integrin receptor complex had any noticeable effect on the formation of capillary tubes. The different results which we obtained in comparison with other investigators (6, 18) may possibly be due to the absence of PMA in our serum-free system utilizing cells sandwiched between fibrin gels. It may be that the simplicity of this system removes unknown factors which may be important for $\alpha_v\beta_3$-mediated capillary tube formation.

Angiogenesis assays frequently make use of complex biologically synthesized matrices such as Matrigel, in which laminin seems to play a predominant role in formation via the expression of three sequences within its structure (23). An Arg-Gly-Asp sequence in the A chain supports cell attachment, while the YIGSR sequence, of the B1 chain induces capillary tube formation. The latter effect may be mediated by a specific interaction with a 67/32-kD endothelial cell surface protein (23). A third region of laminin, the SIKVAV sequence located in the A chain, promotes

migration, invasion of Matrigel, and growth and branching of capillary tubes (19). Thus interactions between matrix proteins and cell surface receptors may underlie the formation of capillary tubes (21). In this regard, it is most interesting that the β15–42 sequence of fibrin has been shown to bind to a 130-kD glycoprotein expressed at the endothelial cell surface (13), and it is conceivable that fibrin II interaction with this receptor may initiate capillary tube formation.

In our experiments, proteolysis of matrix proteins was not required for the formation of capillary tubes, presumably because the monolayers are instantaneously sandwiched between two fibrin gels and do not need to migrate into the gel in order to become surrounded by the matrix. Most importantly, our studies demonstrate that contact of fibrin II with the apical aspect of the endothelial cell monolayer is crucial for the induction of morphogenetic changes. This finding is consistent with other studies that have demonstrated that the interaction of collagen with the apical surface of endothelial cells also induces the formation of capillary tubes (35). However, we observed that exogenous soluble β15–42 peptide inhibited fibrin II–induced tube formation by 45%, while collagen-induced tube formation was unaffected. This finding indicates the specificity of interaction of fibrin with the endothelial cell surface.

In summary, we describe a new in vitro assay for the study of capillary tube formation by endothelial cells sandwiched between two fibrin gels in serum-free medium. The induction of capillary morphogenesis is dependent on the structure of fibrin, with optimal tube formation occurring with fibrin II, minimum tube formation with fibrin I, and complete absence of tube formation with fibrin 325. Excess soluble β15–42 peptide was able to inhibit fibrin II–induced capillary tube formation, suggesting that interaction of the NH₂ terminus of the fibrin II β chain with the apical surface of the endothelial cells plays a principal role in promoting tube formation.

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