Cell Proliferation on Fibrin: Modulation by Fibrinopeptide Cleavage

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Fibrin forms the cohesive network of hemostatic plugs and thrombi, and it also provides the temporary matrix for initial support of healing and revascularization. Because cell proliferation is needed for revascularization after vessel injury, we have characterized structural requirements of fibrin needed to support cell proliferation on fibrin in vitro. Proliferation of cultured human endothelial cells and fibroblasts was measured by \(^{3}H\)-thymidine incorporation on fibrin surfaces varying in structure. Fibrin prepared with thrombin and lacking both fibrinopeptides A and B (desAB fibrin) supported proliferation of both endothelial cells and fibroblasts. In contrast, fibrin prepared with reptilase, which cleaves only fibrinopeptide B, supported significantly less proliferation. Also, fibrin prepared by thrombin treatment of fibrinogen lacking residues \(\beta1-42\) supported only a low level of proliferation at Arg\(^{16}\)-Gly\(^{17}\) of the \(\alpha\) chain and at Arg\(^{44}\)-Gly\(^{45}\) of the \(\beta\) chain, releasing fibrinopeptides A and B, respectively, and exposing new amino terminal ends of the \(\alpha\) and \(\beta\) chains, and these are involved in polymerization. In a previous report, we have shown that fibrinopeptide B (FpB) cleavage and exposure of the \(\beta15-42\) region of the fibrin molecule by thrombin is necessary to stimulate spreading of adherent endothelial cells. DesA fibrin, prepared by cleavage of fibrinopeptide A (FPA) only, or fibrin lacking \(\beta15-42\) supported avid endothelial cell adhesion, but the adherent cells failed to spread. Because interactions of cells with matrix may also modulate proliferation, we have characterized the DNA synthetic rate of endothelial cells and fibroblasts on fibrin surfaces of varying structure.

MATERIALS AND METHODS

**Cell culture.** Primary endothelial cells were procured from human umbilical veins as previously described, and cultured in McCoy’s 5a medium supplemented with 15% fetal bovine serum, 50 \(\mu\)g/mL endothelial cell growth supplement (ECGS) (Biomedical Technologies Inc, Stoughton, MA) and 100 \(\mu\)g/mL heparin (Sigma Chemical Co, St Louis, MO) and passaged twice before use. Primary fibroblasts were obtained by finely mincing newborn human foreskins, digesting them for 30 to 90 minutes in trypsin-EDTA (GIBCO Life Technologies, Grand Island, NY) diluted 1:1 in Hanks’ balanced salt solution at 37°C, and then plating them in a cell culture flask in cell culture medium. Fibroblast cultures were passaged up to five times before use.

**Fibrinogen and fibrin preparation.** Human fibrinogen (grade L) was obtained from Kabivitrum (Franklin, OH), and copurifying factor XIII inactivated and fibronectin were removed as described previously. Purified protease III from *Crotalus atrox* venom was a kind gift of Dr Andrei Budzynski (Temple University, Philadelphia, PA). Fibrinogen 325, lacking residues 1-42 of the \(\beta\) chain was prepared from fibronectin-depleted fibrinogen as described previously. Human thrombin (3,250 U/mg) and reptilase snake venom were obtained from Calbiochem-Behring Corp (La Jolla, CA) and American Diagnostica (Greenwich, CT), respectively. Anthrothrombin, a thrombin derivative in which the active site serine was modified to 2-deoxyxylalanine, rendering it cataytically inactive, was kindly provided by Dr Robert W. Ashton and Dr Harold Scheraga (Cornell University, Ithaca, NY), and was used at an amount equivalent to 2.5 \(U/mL\). The \(\beta15-42\) peptide was purchased as a chain that are also available in fibrin.

**Fibrin formation** within the vasculature is followed by responses of vascular cells to restore normal blood flow and vessel structure. Endothelial cell migration and proliferation are important processes in these responses that are needed for new capillary formation and reendothelialization after injury. Exposure of endothelial cells to fibrin in vitro results in several specific responses including changes in protein secretion, loss of monolayer organization, disruption of cell-cell contacts and migration. Fibrin deposition can also induce new capillary formation in vitro and an angiogenic response in vivo. Cell proliferation in areas of fibrin formation can be stimulated by endothelial cell mitogens including growth factors and thrombin. In addition, a steadily increasing body of evidence suggests that the substrate to which endothelial cells adhere plays an important role in local regulation of capillary development. This regulation is associated with changes in cell shape and control of DNA synthesis.

The endothelial cell response to a fibrin surface is dependent on specific structural features of the fibrin molecule. Adhesion to fibrinogen is mediated through binding of the integrin receptor \(\alpha\) to RGD(S) sites near the carboxyl termini of the \(\alpha\) chain that are also available in fibrin. Fibrin-specific structural features resulting from the action of thrombin on fibrinogen also contribute to endothelial cell responses to a fibrin matrix. Fibrinogen is cleaved by thrombin at Arg\(^{16}\)-Gly\(^{17}\) of the \(\alpha\) chain and at Arg\(^{44}\)-Gly\(^{45}\) of the \(\beta\) chain, releasing fibrinopeptides A and B, respectively, and exposing new amino terminal ends of the \(\alpha\) and \(\beta\) chains, and these are involved in polymerization. In a previous report, we have shown that fibrinopeptide B (FpB) cleavage and exposure of the \(\beta15-42\) region of the fibrin molecule by thrombin is necessary to stimulate spreading of adherent endothelial cells. DesA fibrin, prepared by cleavage of fibrinopeptide A (FPA) only, or fibrin lacking \(\beta15-42\) supported avid endothelial cell adhesion, but the adherent cells failed to spread. Because interactions of cells with matrix may also modulate proliferation, we have characterized the DNA synthetic rate of endothelial cells and fibroblasts on fibrin surfaces of varying structure.
from the peptide and DNA Sequence Facility, Cornell Center of Advanced Technology in Biotechnology (Ithaca, NY). The 14-amino acid thrombin receptor-activating peptide with amino acid sequence SFL was purchased from Peninsula Laboratories, Inc (Belmont, CA), and used at a concentration of 100 μmol/L. Cytochalasin B was purchased from Sigma. Fibrin-coated cell culture wells were prepared using 0.4 mL aliquots of fibrinogen (3 mg/mL) in McCoy’s 5a medium minus fetal calf serum (FCS) to which thrombin (2.5 U/mL), reptilase (0.5 U/mL) used at a lower concentration to avoid unwanted excess proteolytic degradation of the α chains, or a combination was added, mixed, rapidly pipetted into single wells of a 24-well nontissue culture-treated cell culture plate, and aspirated before polymerization resulting in a thin coating of polymerized fibrin. Wells were also coated with fibrinogen at a concentration of 3 mg/mL and human fibronectin (Calbiochem) at concentrations of 30 μg/mL in McCoy’s 5a medium by incubation for 1 hour at room temperature. For studies of cell spreading, a glass coverslip was placed in the cell culture wells before coating. After a 30-minute incubation at room temperature, wells were washed once with McCoy’s 5a medium, then 1 μg/mL D-phenylalanyl-L-prolyl-L-arginyl-chloromethylketone (PPACK) (Bachem California, Torrence, CA), a synthetic, highly specific thrombin inhibitor which covalently binds to the active site of the thrombin molecule, was added to the wells and incubated at room temperature for 30 minutes to inactivate residual thrombin. Wells were then washed three times with McCoy’s 5a medium before addition of cells. Hirudin, a leech-derived high-affinity thrombin inhibitor, was obtained from Sigma and was used in selected experiments.

Measurement of DNA synthetic rate. 3H-thymidine incorporation was used to measure DNA synthesis rather than proliferation per se. At times investigated, this measurement would only indicate entry of cells into S phase of the cell cycle. For measurement of DNA synthesis, 60,000 cells/well (3 × 10⁴ cells/cm²) in McCoy’s 5a medium supplemented with 1% FCS, 50 μg/mL ECGS and 100 μg/mL heparin were placed in fibrin-coated cell culture wells and allowed to adhere and spread for 6 hours. Plating density was chosen so that minimal cell-cell contact occurred during the incubation period. Medium was then removed and replaced with medium containing 1 μCi/mL 3H-thymidine (New England Nuclear, Boston, MA) and allowed to incubate at 37°C for 18 hours. Endothelial cell DNA synthesis assays were conducted in the presence of ECGS and heparin. Duplicate or triplicate wells were plated for measurement of 3H-thymidine incorporation, and duplicate wells were also plated in parallel in the absence of 3H-thymidine for measurement of cell number. At the end of the 18-hour incubation period, nonadherent cells were removed by washing twice with ice-cold phosphate-buffered saline (PBS), then adherent cells were scraped into 250 μL ice-cold PBS and stored at 4°C. To each sample, 250 μL ice-cold 10% trichloroacetic acid (TCA) (Sigma) was added and placed on ice. Acid precipitates were collected on a 0.45-μm filter in a vacuum manifold. Filters were washed four times with 1 mL ice-cold 5% TCA, then once with ice-cold 95% ethanol. Filters were allowed to air dry, and then TCA-precipitable counts per minute (cpm) on each filter was determined after immersion of filter in scintillation fluid.

Cell number determination. The number of cells adherent to wells at the end of each experiment was determined by assay of acid phosphatase activity using wells plated in parallel. Culture medium was removed from the wells, and wells were washed twice with ice-cold PBS. To each well, 125 μL buffer containing 0.1 mol/L sodium acetate (pH 5.5), 0.1% Triton X-100, and 10 mM/L p-nitrophenyl phosphate (Sigma) was added. The plates were then placed at 37°C for 2 hours, at which time 100 μL of each sample was removed and placed onto wells of a 96-well enzyme-linked immunosorbent assay (ELISA) plate. The reaction was stopped with

the addition of 10 μL 1 mol/L sodium hydroxide to each sample. Color development was determined at 410 nm using an ELISA reader. Cell number was estimated based on a standard curve constructed using known numbers of endothelial cells or fibroblasts (obtained by Coulter Counter analysis) plated into wells and allowed to adhere for 2 hours before acid phosphatase assay. Viability of cells was determined by trypan blue exclusion as previously described. Greater than 85% of cells remaining on all fibrin surfaces were viable after 18-hour incubation.

Cell spreading. Cell-spreading status was assessed using cells plated in parallel onto fibrin-coated glass coverslips. Cells on coverslips were fixed for 20 minutes in 3.7% formaldehyde in PBS, permeabilized for 15 minutes in 0.5% Triton X-100 in PBS, then stained for F-actin with rhodamine-phalloidin (Molecular Probes Inc, Eugene, OR) at a dilution of 1:10 for 30 minutes. Coverslips were then mounted onto glass slides using Fluoromount (Fisher Scientific, Pittsburgh, PA), and spreading was evaluated by fluorescence microscopy.

Fibrin sample preparation and gel electrophoresis. Fibrin was prepared as described above, except that after addition to cell culture wells, excess fibrin solution was not aspirated but was allowed to polymerize. Fibrin samples were incubated for 18 hours at 37°C in the absence of cells before addition of sample denaturant. Polyepitide chain composition of fibrin was analyzed after disulfide bond reduction on a 7% SDS polyacrylamide gel using the method of Weber and Osborn. Gels were stained using Coomassie Blue.

Statistical analysis. P values were obtained using a paired or unpaired one-tailed Student’s t-test.

RESULTS
DNA synthesis in human umbilical vein endothelial cells was measured on fibrin coatings of varying structure. DesAB fibrin was prepared using thrombin that cleaves both fibrinopeptides A and B; desA fibrin was prepared using reptilase, which cleaves only FPA. In addition, because reptilase treatment can cause proteolysis of the fibrin α chain, desAB fibrin was also prepared with the addition of reptilase to control for any inhibitory effects of unwanted proteolysis. All fibrin coatings were treated with PPACK, an inhibitor of the thrombin active site, before addition of endothelial cells to inactivate any residual thrombin that remained on the fibrin surface after extensive washing. Results presented in Fig 1, taken from a single representative experiment, show cpm of 3H-thymidine incorporated into endothelial cells plated on uncoated wells and on wells coated with fibronectin, fibrinogen, desAB fibrin, desA fibrin, and desAB fibrin with the addition of reptilase. Also shown are estimates of cell number, which were determined by assay of acid phosphatase activity in the well at the end of the 18-hour incubation period and which showed that the number of cells remaining on the various adhesive surfaces was variable. Because plastic was nontissue culture treated and, therefore, did not provide an adequate adhesive surface, 3H-thymidine incorporation and number of cells remaining on uncoated wells were minimal. Both 3H-thymidine incorporation and cell adherence were highest on wells coated with fibronectin and unclotted fibrinogen. In agreement with the prior observation that cell adhesion was the same on desAB and desA fibrin, the number of cells remaining on the various fibrin surfaces was similar. However, 3H-thymidine incorporation
Fig 1. Endothelial cell proliferation on various adhesive substrates. Endothelial cells were plated onto uncoated, nontissue-culture-treated wells or wells coated with fibronectin, fibrinogen, desAB fibrin, desA fibrin, and desAB fibrin with the addition of reptilase. ³H-thymidine incorporation cpm (□) was determined in each of three wells in a single experiment, mean and standard error of which are shown. The number of adherent cells remaining at the end of the 18-hour incubation period (■) was determined in a well plated in parallel using an acid phosphatase assay.

was reduced on desA fibrin, indicating that the DNA synthetic rate of cells adherent to this surface was less than on desAB fibrin.

To allow comparison among experiments and to control for variations in cell number, subsequent results are expressed as cpm per cell and are normalized to results obtained on desAB fibrin. Normalized results obtained from five to eight experiments using both endothelial cells and fibroblasts are presented in Fig 2. Endothelial cell DNA synthetic rate on desA fibrin was $0.37 \pm 0.03$ compared with $1.0 \pm 0.03$ measured on desAB fibrin ($P < 1 \times 10^{-4}$). Fibroblast DNA synthesis was also reduced on desA fibrin compared with desAB fibrin ($0.54 \pm 0.04$ and $1.0 \pm 0.07$, respectively; $P = 0.004$). There was a decrease in endothelial cell DNA synthesis on desAB fibrin with added reptilase ($0.85 \pm 0.07$; $P = 0.03$), but the difference between DNA synthesis on desA fibrin and on desAB fibrin containing reptilase remained highly significant ($P = 3 \times 10^{-4}$). No decrease in fibroblast DNA synthesis was observed because of the presence of reptilase in desAB fibrin ($1.00 \pm 1$; $P = 0.5$).

Spreading of endothelial cells and fibroblasts on the fibrin surfaces was quantitated by microscopic observation after staining of the cells’ actin-containing cytoskeleton using rhodamine phalloidin. Actin-containing microfilaments are responsible for force transduction within cells and, therefore, play an important role in determining cell shape.⁶ On desAB fibrin, most endothelial cells and fibroblasts had a flattened morphology with parallel arrays of microfilaments traversing the cell cytoplasm. On desA fibrin, the majority of both endothelial cells and fibroblasts exhibited a rounded morphology with a reduced cell diameter and no formation of microfilaments. Cells were scored by microscopic observation as spread if they contained such arrays of microfilaments and as round if no such actin polymerization was seen. Fifty to one hundred cells were scored in each experimental condition. As is shown in Table 1, cell spreading on desAB fibrin was greater than spreading on desA fibrin. Shown in Fig 3 are photos of representative cells displaying the two types of spreading behavior on desAB and desA fibrin.

Samples for gel analysis of fibrin structure were incubated at 37°C for 18 hours in the absence of cells (Fig 4) and, therefore, do not reflect any possible modifications of the fibrin surface made by the adherent cells. DesA fibrin (Fig 4, lanes 2 and 3) exhibited cleavage of fibrinopeptide A, but not B, as shown by the slightly increased migration of the $\alpha$ chain as compared with fibrinogen (Fig 4, lane 1). Reptilase caused some degradation of the carboxyl terminus of the $\alpha$ chain as shown by increased migration of this chain and the appearance of lower-molecular-weight forms. This was also apparent in the desAB fibrin containing reptilase (Fig 4, lane 5). DesAB fibrin exhibited faster migration of both the $\alpha$ and $\beta$ chains caused by cleavage of both FPA and FPB (Fig 4, lane 4). The DNA synthetic capacity of the cells may have been increased by the mitogenic effects of thrombin present in residual amounts on desAB fibrin. Although the active site of any residual, fibrin-bound thrombin in desAB fibrin is blocked using PPACK, the presence of the thrombin exosite could still play a stimulatory role in cell proliferation.

To investigate such involvement, desA fibrin was prepared in the presence of 2-dehydroxylalanine with preservation of the ex-

### Table 1. Percent Cell Spreading on desAB and desA Fibrin

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<th>EC</th>
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<tr>
<td>desAB</td>
<td>79 ± 6</td>
<td>71 ± 12</td>
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<td>desA</td>
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Results shown are mean ± standard error.

Abbreviations: EC, endothelial cell; HFF, human foreskin fibroblast.
Fig 3. Fluorescence microscopy of endothelial cells and fibroblasts on desAB and desA fibrin. Cells were plated onto fibrin-coated glass coverslips for 18 hours, then fixed and stained with rhodamine phalloidin to visualize the actin-containing cytoskeleton. On desAB fibrin, both endothelial cells (a) and fibroblasts (b) exhibited a flattened morphology with actin filament bundles traversing the cell cytoplasm in parallel arrays. On desA fibrin, endothelial cells (b) and fibroblasts (c) exhibited a rounded morphology with greatly decreased cell diameter and no evidence of actin polymerization. All photographs were taken at the same magnification; bar, 20 μm.

The absence of a native fibrinopeptide B (FPB) in desA fibrin may be responsible for the enhanced DNA synthesis of endothelial cells and fibroblasts on desAB fibrin as compared with that on desA fibrin. This hypothesis was tested by exposing cells plated on desA fibrin to thrombin receptor activating peptide (SFLL) to determine if DNA synthesis could be increased by stimulating the endothelial cell thrombin receptor. Enzymatically active thrombin could not be used for this experiment because cleavage of FPB would occur, stimulating proliferation independently. SFLL at a concentration of 100 μmol/L did not stimulate proliferation or spreading of endothelial cells on desA fibrin.

The results presented above suggest that the enhanced DNA synthesis of endothelial cells and fibroblasts on desAB fibrin as compared with that on desA fibrin is caused by cleavage of fibrinopeptide B with resultant exposure of the β15-42 region of the fibrin molecule. To investigate this further, we used protease III purified from Crotalus atrox venom, which cleaves fibrinogen between residues 42 and 43 of the Bβ chain, thereby removing the amino-terminal 42 residues including the 15-42 peptide. Such cleaved fibrinogen (fibrinogen_{325}) was then treated with thrombin to produce fibrin_{325} in which the β15-42 region was absent. In gel analysis (Fig 6), this altered fibrinogen (Fig 6, lane 2), when compared with native fibrinogen (Fig 6, lane 1), exhibited faster migration of the β chain due to the absence of the Bβ 1-42 peptide. After thrombin exposure there was no shift in migration of the β chain of fibrin_{325} (Fig 6, lane 4) as is seen after thrombin cleavage of native fibrinogen (Fig 6, lane 3), but the expected increase in migration of the α chain occurred. DNA synthesis of endothelial cells on fibrin_{325} was lower when compared with that on unmodified desAB fibrin (.21 ± .04 v 1.0 ± .03; P = .0003) (Fig 7).

Spreading on fibrin_{325} was also reduced (37%) when compared with spreading on normal desAB fibrin (93%).

Endothelial cells plated on desA fibrin were exposed to the thrombin receptor activating peptide (SFLL) to determine if DNA synthesis could be increased by stimulating the endothelial cell thrombin receptor. Enzymatically active thrombin could not be used for this experiment because cleavage of FPB would occur, stimulating proliferation independently. SFLL at a concentration of 100 μmol/L did not stimulate proliferation or spreading of endothelial cells on desA fibrin.
Ten-microgram samples were electrophoresed on a 1806 fader migration with Coomassie Blue. The position of each polypeptide chain is indicated, identified by comparison with a standard of fibrinogen (lane 1). Lane 2, desA fibrin, showing faster migration of the α chain because of cleavage of FPA only. There is some degradation of the α chain caused by proteolysis resulting in decreased staining of the α chain as well as the appearance of lower-molecular-weight forms. Lane 3, desA fibrin prepared with the addition of catalytically inactive anhydrothrombin (in an amount equivalent to 2.5 U/mL) exhibiting faster migration of the α chain and no change in migration of the β chain. Lane 4, desAB fibrin exhibiting faster migration of both the α and β chains because of cleavage of both fibrinopeptides A and B. Lane 5, desAB fibrin prepared with the addition of reptilase, showing increased migration of both the α and β chains as well as degradation of the α chain equivalent to that seen in desA fibrin. In each fibrin sample, the γ chain was monomeric indicating that cross-linking to a dimeric form had not occurred.

To determine if exposure to the β15-42 peptide could induce proliferation in the absence of cell spreading, endothelial cells plated on desA fibrin were exposed to the soluble β15-42 peptide (1 mmol/L). This exposure did not result in increased DNA synthesis nor did enhanced cell spreading occur (Fig 8).

To further explore the influence of cell spreading status on modulation of DNA synthesis α fibrin, endothelial cells were plated on desAB fibrin in the presence of cytochalasin B (10 μmol/L) to inhibit cell spreading. Cytochalasin B treatment effectively inhibited spreading on this surface (Fig 8B), and DNA synthesis of endothelial cells adherent to desAB fibrin under these conditions was also significantly inhibited (Fig 8A).

**DISCUSSION**

Rates of DNA synthesis of both human umbilical vein endothelial cells and human foreskin fibroblasts were measured on fibrin surfaces varying in structure with regard to exposure of the β15-42 region of the fibrin molecule. Both cell types were actively proliferative, as determined by 3H-thymidine incorporation, on desAB fibrin, but DNA synthesis was greatly reduced on desA fibrin. DNA synthesis of cells on fibrin 325, formed by thrombin cleavage of fibrinogen lacking the Bβ1-42 region, was also reduced when compared with that on fibrin formed by thrombin cleavage of native fibrinogen. These results indicate that exposure of the β15-42 region is an important determinant in the ability of fibrin to support cell proliferation. In previous reports, we have shown that exposure of this region of the β chain is necessary for endothelial cell as well as platelet spreading to occur on fibrin surfaces. Erban and Wagner have identified a nonintegrin receptor on endothelial cells that binds specifically to the β15-42 region of fibrin and could potentially be involved in mediating specific cell responses to fibrin.

A close relationship between cell shape and control of cell growth has been found in several cell culture systems and suggests that the matrix on which cells grow can act as a local regulator of proliferation. For example, the rate of proliferation of fibroblasts and endothelial cells varies directly with the degree of cell spreading in a system in which adhesiveness was controlled by growing cells on plastic surfaces treated with varying concentrations of poly (2-hydroxyethylmethyl methacrylate). DNA synthesis in capillary endothelial cells increases exponentially with linear increases in projected cell area and, on fibronectin, varies with cell-surface integrin binding and resistance to cytoskeletal tension. However, the exact nature of the relation generated between cellular shape and proliferation remains unclear. Most studies point to a mecanochemical mechanism whereby occupancy of adhesive receptors alone is not sufficient to induce DNA synthesis and which occurs only with spreading of cells and the generation of tensile force. The...
MODULATION OF CELL PROLIFERATION ON FIBRIN

Fig 6. SDS polyacrylamide gel electrophoresis of fibrinogen, fibrin, fibrinogen235, and fibrin235. Samples containing 10 μg were electrophoresed on SDS 7% polyacrylamide gels after disulfide-bond reduction and were stained with Coomassie Blue. Fibrinogen was prepared with protease III from Crotalus atrox venom, which results in cleavage of the Bβ chain between residues 42 and 43. Fibrinogen235 (lane 2) contained a predominance of Bβ chain lacking residues 1-42, and, therefore, this chain exhibited faster migration than native fibrinogen (lane 1). Fibrinogen235 contained a small amount of residual, uncleaved Bβ chain. Fibrin235 (lane 4) prepared with thrombin exhibited more rapid migration of the α chain than fibrin (lane 3) because of cleavage of FPB. There was also increased migration of the residual, uncleaved β chain because of cleavage of FPB. No change in migration of the β chain derivative lacking residues 1-42 (ββ) occurred after thrombin treatment.

tensile force can expand the area of cell surface contact and stimulate DNA synthesis in several ways, including alteration in cytoskeletal polymerization, changes in stretch-activated ion channels in the plasma membrane, or nuclear enlargement and release of physical restraints on nuclear function. Changes in intracellular pH brought about by integrin receptor occupancy and resulting changes in cell shape have been linked to control of cell proliferation. This change in pH results from activation of a Na+/H+ antiporter on the cell surface.

DNA synthetic rates of cells plated on fibrin surfaces correlated with the spreading activity (Figs 2 and 3 and Table 1), and we hypothesize that the effect of fibrin structure on cell shape and DNA synthesis are related. Control of cell proliferation on fibrin may also be governed by a mechanochemical mechanism with a specific structure of the polymerized fibrin required. Several results presented here favor this hypothesis. DNA synthesis was low on desAB fibrin in the presence of cytochalasin B, which inhibits microfilament polymerization and thereby prevents cell spreading (Fig 8).

The β15-42 peptide has been shown to support cell spreading only when present in the intact fibrin molecule or when immobilized to a surface after conjugation to ovalbumin. Exposure of endothelial cells to soluble β15-42 peptide at a concentration that can induce von Willebrand factor release from Weibel-Palade bodies did not result in cell spreading and did not stimulate DNA synthesis on desA fibrin (Fig 8). Therefore, cell spreading and DNA synthesis induced by exposure to the β15-42 region of fibrin are not parallel, unrelated processes, but proliferation is likely dependent on the capacity of the cells to spread on the fibrin surface.

Thrombin is a potent inducer of fibroblast mitogenesis and stimulates cell proliferation after cleavage of a cell-surface thrombin receptor by a novel tethered ligand mechanism. Thrombin has also been shown to stimulate endothelial cell mitogenesis and it enhances incisional wound healing and neovascularization in vivo. Therefore, thrombin could play an important role in stimulating endothelial cell proliferation in areas of fibrin formation. However, several control experiments eliminated the possibility that enhanced DNA synthesis observed on desAB fibrin was the result of residual fibrin-bound thrombin. Thrombin bound to fibrin can be effectively inhibited using PPACK, a potent thrombin inhibitor that acts through covalent binding to the thrombin active site. All fibrin surfaces were washed with PPACK in molar excess to the amount of thrombin used in fibrin formation before addition of cells. Other regions of the thrombin molecule could potentially be involved in stimulating endothelial cell proliferation, including an RGD site located at residues 187-189 of the thrombin β chain. Thrombin also contains an anion binding exosite that is required for activation of the cloned thrombin receptor and for platelet activation by thrombin. Hirudin is a highly specific, leech-derived thrombin inhibitor that binds to both the thrombin-active site and the thrombin exosite and can bind...
was measured by incorporation of \(^3\)H-thymidine on desAB fibrin, and peptide (SFLL) and p15-42 peptide. (A) Endothelial cell proliferation cell spreading. Cell proliferation was also measured on desA fibrin, and on desAB fibrin in the presence of 10 \(\mu\)mol/L cytochalasin B to inhibit cell spreading. Cell proliferation was also measured on desA fibrin, and on desA fibrin in the presence of 100 \(\mu\)mol/L of the thrombin receptor activating peptide, SFLL, and in the presence of 1 \(\mu\)mol/L of the \(\beta 15-42\) peptide. Results are expressed as cpm per cell normalized to values obtained on desAB fibrin. Mean values and standard errors are shown. (B) Cell spreading status was assessed on cells plated in parallel on fibrin-coated glass coverslips that were stained by fluorescence using rhodamine phalloidin by microscopic observation of 50 to 100 cells per condition. Spreading results are expressed as mean values obtained from five experiments.

Fig 8. Endothelial cell proliferation and spreading on desAB fibrin ± cytochalasin B and on desA fibrin ± thrombin receptor activating peptide (SFLL) and \(\beta 15-42\) peptide. (A) Endothelial cell proliferation was measured by incorporation of \(^3\)H-thymidine on desAB fibrin, and on desAB fibrin in the presence of 10 \(\mu\)mol/L cytochalasin B to inhibit cell spreading. Cell proliferation was also measured on desA fibrin, and on desA fibrin in the presence of 100 \(\mu\)mol/L of the thrombin receptor activating peptide, SFLL, and in the presence of 1 \(\mu\)mol/L of the \(\beta 15-42\) peptide. Results are expressed as cpm per cell normalized to values obtained on desAB fibrin. Mean values and standard errors are shown. (B) Cell spreading status was assessed on cells plated in parallel on fibrin-coated glass coverslips that were stained by fluorescence using rhodamine phalloidin by microscopic observation of 50 to 100 cells per condition. Spreading results are expressed as mean values obtained from five experiments.

to and inhibit clot-bound thrombin. Inclusion of a hirudin wash before cell plating did not alter proliferation of cells on desAB fibrin. DesA fibrin was prepared in the presence of catalytically inactive anhydrothrombin in which the active site has been altered by modification of the native serine to 2-dehydroxyalanine with no change in the exosite or other regions of the molecule. Cell proliferation was the same on desA fibrin prepared with or without addition of anhydrothrombin (Fig 5). Furthermore, proliferation of cells was low on fibrin, even though active thrombin was used in its preparation (Fig 7). Lastly, mimicking thrombin exposure of cells plated on desA fibrin, using the thrombin receptor activating peptide (SFLL) at a concentration reported to result in responses of cells similar to those induced by thrombin, did not stimulate DNA synthesis (Fig 8). Therefore, thrombin likely could not exert a mitogenic response in the absence of cell spreading.

Prolonged exposure of fibrin to reptilase, as in our experiments, resulted in some \(\alpha\) chain degradation in addition to specific cleavage of fibrinopeptide A (Fig 4). Because an RGD sequence is present at the C-terminal fibrin \(\alpha\) chain, this degradation could potentially have an inhibitory effect on cell proliferation through decreased adhesivity independent of the \(\beta 15-42\) region. However, we have observed no significant decrease in proliferation of cells on fibrin formed using a combination of both thrombin and reptilase compared with that prepared with thrombin alone (Fig 2), and cells spread adequately on such a fibrin surface.

Decreased proliferation was not the result of reduced cell adhesion to desA fibrin, because there was no significant difference in numbers of cells adherent to desA or desAB fibrin after the 18-hour incubation period. Also, the decreased proliferation of endothelial cells on desA fibrin was not a result of loss of cell viability because greater than 85% of adherent cells on all fibrin surfaces were found to be viable by trypan blue exclusion.

Fibrin, in addition to providing a hemostatic matrix, also supports initial cellular processes required for healing and vessel repair. Upon injury to the vasculature, vessel wall cells and platelets are capable of responding directly to fibrin formation. These cells are normally exposed to high concentrations of fibrinogen in blood, and responses to fibrin require its distinct recognition. This recognition may depend on unique structural features of fibrin. One specific site results from the thrombin-induced cleavage of fibrinopeptide B from fibrinogen that exposes a fibrin-specific region including the 15-42 sequence of the \(\beta\) chain. This region, at the new amino terminus of the \(\beta\) chain, plays an important role in stimulating vascular cell responses including endothezial cell release of von Willebrand factor as well as spreading of both endothelial cells and platelets. In the present study we have shown that cell proliferation, an important process involved in revascularization, also requires exposure of this fibrin-specific site. Therefore, modulation of cell proliferation by specific structural features of fibrin is a unique example of substrate control of cell adhesive and growth processes regulated by specific proteolytic modification of adhesive protein substrates at sites of tissue injury.

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