An Arg-Gly-Asp Sequence Within Thrombin Promotes Endothelial Cell Adhesion

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Abstract. Thrombin, in addition to its central role in hemostasis, possesses diverse cellular bioregulatory functions implicated in wound healing, inflammation, and atherosclerosis. In the present study we demonstrate that thrombin molecules modified either at the procoagulant or catalytic sites induce endothelial cell (EC) adhesion, spreading, and cytoskeletal reorganization. The most potent adhesive thrombin analogue (NO_2-α-thrombin) was obtained by nitration of tyrosine residues. The cell adhesion promoting activity of NO_2-α-thrombin was blocked upon the formation of thrombin-antithrombin III (ATIII) complexes and by antiprothrombin antibodies, but was unaffected by hirudin. Arg-Gly-Asp-containing peptides, fully inhibited EC adhesion to NO_2-α-thrombin, while synthetic peptides corresponding to thrombin "Loop B" mitogenic site and the thrombin-derived chemotactic fragment "CB67-129", were uneffective. Immunofluorescence studies indicated that EC adhesion to NO_2-α-thrombin was followed by cell spreading, actin microfilament assembly, and formation of focal contacts. By the use of specific antibodies, the vitronectin (vn) receptor ($\alpha_v\beta_3$) was found to be localized in clusters upon cell adhesion to NO_2-α-thrombin. An anti $\alpha_v\beta_3$ antibody blocked EC adhesion and spreading while antifibronectin (fn) receptor ($\alpha_5\beta_1$) antibodies were uneffective. While native thrombin exhibited a very low cell attachment activity, thrombin that was incubated at 37°C before coating of plastic surfaces induced EC attachment and spreading. We propose that under certain conditions the naturally hindered RGD domain within thrombin is exposed for interaction with $\alpha_v\beta_3$ on EC. This in turn promotes cell adhesion, spreading, and reorganization of cytoskeletal elements, which may altogether contribute to repair mechanisms in the disturbed vessel wall. This study defines a new biological role of thrombin and characterizes a new recognition mechanism on EC for this molecule.

THE integrity of the vascular system is maintained by factors that mediate the attachment and growth of endothelial cells (ECs).[1] The luminal surface of blood vessels is composed of closely apposed ECs providing a nonthrombogenic surface designed to maintain blood fluidity (31). Upon vascular injury, exposure of blood to thrombogenic subendothelial structures stimulate rapid generation of a hemostatic plug consisting of platelets and fibrin (2, 33, 41).

Thrombin (E.C. 3.4.21.5), the final activation product of the clotting cascade, is responsible for converting fibrinogen to fibrin monomers that polymerize spontaneously to form a typical clot mesh. In addition to its major role in hemostasis, thrombin, a multifunctional serine protease, degrades various constituents of the extracellular matrix (ECM) (28) and induces cellular responses such as proliferation and chemotaxis (3, 4, 6, 9, 36). We have recently demonstrated that thrombin binds to the subendothelial ECM through a short anchorage binding site, leaving the majority of the molecule functional and available for cellular interactions (5). ECM-immobilized thrombin was found to be protected from inactivation by the circulating inhibitor antithrombin III (ATIII). Thus, thrombin when sequestered by the ECM may exhibit a localized, long-acting stimulation of surrounding tissues (6). Amino acid analysis of thrombin B-chain reveals the presence of an Arg-Gly-Asp-Ala sequence at residues 187–190 (26). Because thrombin is ubiquitous to injury sites, we wondered whether it can participate in EC adhesion and thus contribute to repair mechanisms of vascular lesions. In the present study we demonstrate that thrombin preparations modified at either the procoagulant or catalytic sites induces

1. *Abbreviations used in this paper*: ABAE, adult bovine aortic endothelial cells; ATIII, antithrombin III; BAEC, bovine aortic endothelial cells; EC, endothelial cells; ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cells; $MeSO_2$, methysulfonyl fluoride; TLCK, *N*-α-tosyl-L-lysylchloromethyl-ketone.

EC adhesion, spreading and reorganization of thick microfilament bundles. This EC adhesion was inhibited by RGD containing peptides, was accompanied by clustering of vitronectin receptors in focal contacts, and was inhibited by antibodies directed against the vitronectin receptor. We propose that upon certain modifications of thrombin, the naturally hindered RGD domain is exposed for interactions with the vitronectin receptor ($\alpha v\beta_3$) on the EC surface.

Materials and Methods

Cells

Cloned populations of adult bovine aortic ECs (ABAE) were isolated as described (30). Cells were cultured in DME (1 g glucose/liter) containing 10% bovine calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) (Gibco Laboratories, Grand Island, NY) at 37°C in 10% CO_2 humidified incubators. Partially purified, brain-derived bFGF (100 ng/ml) was added every other day during the phase of active cell growth. Human umbilical vein ECs (HUVEC) were obtained and cultured as described (15). The cells were grown to confluence in plastic flasks (Falcon Labware, Becton Dickinson & Co., Oxnard, CA) in medium 199 (M199) supplemented with 20% FCS and antibiotics. Cells were cultured at 37°C in 5% CO_2 humidified incubators and the medium replaced three times a week. Experiments with HUVEC were carried out between the first and fifth passage and with ABAE at passage 5–30. Cells were dissociated with 0.05% trypsin/0.02% EDTA in PBS and subcultured at a split ratio of 1:5. ECs were characterized by indirect immunofluorescence using rabbit anti human factor VIII antigen (Behringwerke Ag, Mailburg, Federal Republic of Germany).

Human Thrombin Preparations

Highly purified human α-thrombin was prepared from fraction IV paste, evaluated for purity, and characterized as described previously (22, 23). Nitration of tyrosine residues to yield NO_2-α-thrombin was performed using tetranitromethane at pH 7.8 as described (36). Specific activity was reduced from 3,975 to 14 clotting U/mg protein and the *p*-nitrophenyl-*p*-guanidinobenzoate-titratable active sites were reduced from 84.4 to 66%. The derivatives α-thrombin, methylsulfonyl fluoride ($MeSO_2$)-α-thrombin, TLCK-α-thrombin and exosite affinity-labeled thrombin were prepared as described previously (6). Modified thrombin preparations were dialyzed extensively against 0.75 M NaCl, and stored frozen at −70°C. Thrombin chemotactic fragment ("CB67-129") and the synthetic peptides corresponding to residues 367–380, 371–380, and 375–380 of thrombin B-chain, as well as various constructs of this region, were kindly provided by Dr. George D. Wilner (American Red Cross, Albany, NY).

Preparation of Coated Surfaces

Thrombin and various modified thrombin preparations were diluted to 10–50 μl/ml in PBS containing 0.1% BSA and adsorbed onto the surface of 16-mm wells of 4-well plates (Nunc, Roskilde, Denmark) by incubation at 4°C overnight. Unbound thrombin was removed and the dishes washed three times with PBS. To determine the amount of thrombin adsorbed to the surface of the dish, thrombin was iodinated as described (27) and the amount of protein adsorbed to the tissue culture plastic determined by γ-counting of radioactive material solubilized with 1 N NaOH. In few experiments human plasma vitronectin, purified as described (42), and human plasma fibronectin, purified by affinity chromatography on gelatin-Sepharose (20) were used to coat culture dishes and glass coverslips, as described. Glass coverslips (10 mm diameter) were cleaned by sonication for 10 min, immersed in ethanol/ether (1:1[vol/vol]) for 2 h and dried before placing in 4-well tissue culture plates (Falcon Labware, Oxnard CA). The coverslips were coated with 0.3 ml of either vitronectin (7 μg/ml), α-thrombin, or NO_2-α-thrombin (100 μg/ml) in PBS containing Ca^{2+} and Mg^{2+}, pH 7.4, for 2 h at 37°C. Residual protein binding sites on coverslips were saturated by further incubation (30 min, 37°C) with 1% BSA (fatty acid free; Sigma Chemical Co., St. Louis, MO) in Ca^{2+},Mg^{2+} containing PBS. Coverslips were then washed twice with PBS before cell seeding.

Preparation of ECM

Corneal endothelial cells were plated at an initial density of 4×10^4 cells per 35-mm dish (Falcon Labware) and maintained as described for aortic EC except that 5% dextran T-40 (Pharmacia, Uppsala, Sweden) was included in the growth medium to obtain a thicker ECM layer. 6–8 d after the cells reached confluency the subendothelial ECM was exposed by dissolving (3 min, 22°C) the cell layer with a solution containing 0.5% Triton X-100 and 20 mM NH_4OH in PBS (25). The ECM remained intact, firmly attached to the entire area of the tissue culture dish and free of nuclear debris.

Antibodies

Antibodies recognizing the vitronectin receptor ($\alpha_v\beta_3$) and the fibronectin receptor ($\alpha_5\beta_1$) were used. $\alpha_v\beta_3$ antibodies were the following. (*a*) A purified human platelet GpIIIa rabbit antiserum that recognizes integrin β_3 (24, 37) was prepared in our laboratory as previously described (15). (*b*) mAb LM142 directed against α_v chain. (*c*) mAb LM609 directed to α_vB_3 complex. These mAbs were kindly donated by Dr. D. Cheresh (Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, CA) (11). The anti-$\alpha_5\beta_1$ antibodies were: (*a*) a goat antiserum directed to purified $\alpha_5\beta_1$ prepared in our laboratory and characterized elsewhere (12); (*b*) a rabbit antiserum to the α_5 cytoplasmic domain, kindly donated by Dr. J. A. McDonald (Washington University, School of Medicine, St. Louis, MO); and mAb (BIE5) directed to α_5 chain was obtained from Dr. C. Damsky (Department of Anatomy, University of California San Francisco, CA). Preimmune goat and rabbit sera and a mAb (10E3) to human platelet GpIIb-IIIa, which does not recognize endothelial cells (10) (kindly donated by Dr. B. S. Coller, State University of New York at Stony Brook, NY) were used as negative controls. The localization of vinculin was assayed with a mouse mAb to chicken gizzard vinculin cross reacting with the mammalian vinculin (clone VIN-11-5; code 6501; Bio Makor, Rehovot, Israel). Antiprothrombin antibodies were purchased from DAKOPATTS (Copenhagen, Denmark).

Attachment Assay

Confluent endothelial cells were dissociated with trypsin-EDTA solution washed once in growth medium, and resuspended in DME containing 0.2% BSA. Cells (1.3×10^5 cells/well) were added to each protein-coated well and incubated at 37°C for 2 h. The plates were washed three times with PBS and the firmly attached cells were fixed with 3% paraformaldehyde. Fixed cells were rinsed with 0.1 M borate buffer (pH 8.5), stained (10 min at 22°C) with 0.1 ml methylene blue (1% in 0.1 M borate buffer, pH 8.5) per well, and washed four times in borate buffer. This procedure removed practically all noncell-bound dye. Specific cell incorporated methylene blue was dissolved with 0.1 N HCl (0.2 ml/well, 40 min, 37°C) and determined by its absorbance at 600 nm. Uptake of methylene blue is linearly correlated to the number of viable cells (29).

To determine whether protein synthesis and/or secretion participate in thrombin induced cell attachment, ECs were plated with or without 5 ng/ml emetine (Sigma Chemical Co.) or 0.7 ng/ml monensin (Sigma Chemical Co.) to inhibit protein synthesis and secretion, respectively (14, 17, 41). Cells were treated with the drugs 1 h before plating on specific substrates and kept in the presence of the drugs during the adhesion assay. Each point is the average of triplicate wells and standard error did not exceed ±5% of the mean. Each experiment was performed at least three times yielding comparable results and the variation between different determinants did not exceed 15%.

RGD-containing Peptides

The synthetic tetra-, penta-, and hexapeptides (GRGD, GRGDS, GRGDSP, GRGESP) were obtained from Peninsula Laboratories, Inc. (Belmont, CA). In some experiments ECs were plated in the presence of these synthetic peptides and analyzed for the extent of cell attachment.

Fluorescence Studies

For immunofluorescence experiments, detached cells were washed once in growth medium resuspended in serum-free M199 medium at a concentration of $1.5–2 \times 10^5$ cells/ml, and 0.3 ml of the cell suspension was seeded on each coated coverslip. After the indicated time of incubation at 37°C, the wells were washed three times with 1 ml PBS containing Ca^{+2} and Mg^{+2}. The coverslip-attached cells were fixed in 3% formaldehyde (from paraformaldehyde) in PBS, pH 7.6, containing 2% sucrose, for 5 min at room temperature. After rinsing in PBS, cells were permeabilized by soaking the coverslips for 3–5 min at 0°C in Hepes-Triton X-100 buffer (20 mM Hepes, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, and 0.5% Triton X-100). This procedure of fixation and permeabilization has been

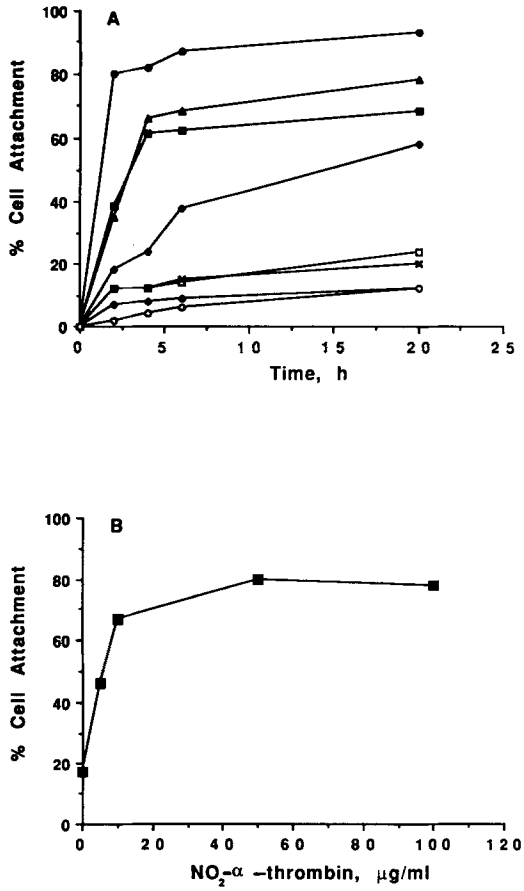

Figure 1. EC adhesion to various modified thrombin preparations. (*A*) Time course. Dishes were coated with different thrombin analogues (10–50 μg/ml) in PBS containing 0.1% BSA. EC (1.3 × 10^5 cells/well) were added and unattached cells removed at various time intervals after seeding. Substrates: (●) NO_2-α-thrombin, (▲) Exo site affinity label-thrombin, (■) $MeSO_2$-thrombin, (♦) γ-thrombin, (□) TLCK-α-thrombin, (×) DIP-α-thrombin, (◇) α-thrombin, (○) BSA. (*B*) Dose dependence. 4-well plates were coated with increasing concentrations of NO_2-α-thrombin. ECs (1.3 × 10^5 cells/well) were seeded and unattached cells removed after 2 h incubation at 37°C. The degree of cell attachment, expressed as percentage of attached cells relative to the number of cells seeded, was evaluated by the uptake of methylene blue, as described in Materials and Methods.

successfully used in various studies with EC (15–17). To show F-actin in the process of cell spreading and microfilament organization, fixed and permeabilized cells were stained with 2 μg/ml rhodamine-labeled phalloidin (R-PHD, a kind gift of Dr. T. Wieland, Max Planck Institute for Experimental Medicine, Heidelberg, FRG) for 30 min at 37°C. Indirect immunofluorescence staining was performed as reported (15–17). Briefly, the primary antibody was layered on fixed an permeabilized cells and incubated in a humid chamber for 30 min. After rinsing in PBS-0.2% BSA, coverslips were incubated with the appropriate rhodamine-tagged secondary antibody (DAKOPATTS) for 30 min at 37°C in the presence of 2 μg/ml of fluorescein-labeled phalloidin. Coverslips were then mounted in 50% glycerol-PBS. Observations were carried out in a Zeiss Axiophot photomicroscope equipped for epifluorescence and interference refection microscopy. Fluorescence images were recorded on Kodak T-Max 400 films exposed at 1000 ISO and developed in Kodak T-Max developer for 10 min at 20°C.

Results

Endothelial Cell Adhesion to Thrombin

Fig. 1 *A* demonstrates the attachment of bovine aortic endothelial cells (BAEC) to α-thrombin and to a variety of thrombin species that were chemically modified to alter thrombin procoagulant or esterolytic functions. NO_2-α-thrombin was found to be the most potent adhesive substratum, inducing attachment of ~80% of the cells within 2 h. Modified thrombin preparations inactivated by the fibrinopeptide exosite affinity label (Exo-α-thrombin) and the mesyl-conjugated active site serine ($MeSO_2$-α-thrombin) were highly active in promoting BAEC attachment. γ-Thrombin, lacking the procoagulant site of thrombin, was also active. In contrast, the native enzyme (α-thrombin) and the esterolytically inactive forms, *N*-α-tosyl-L-lysylchoromethyl-ketone (TLCK)-α-thrombin and diisopropylfluorophosphate-α-thrombin, were poorly active and the level of cell attachment was only slightly above that observed on noncoated surfaces.

To further investigate the requirements for thrombin-mediated induction of EC adhesion, the following experiments were performed using NO_2-α-thrombin as the adhesive substratum. Fig. 1 *B* demonstrates that cell attachment was dependent on the concentration of NO_2-α-thrombin used to coat the dishes, reaching saturation at 20–50 μg/ml.

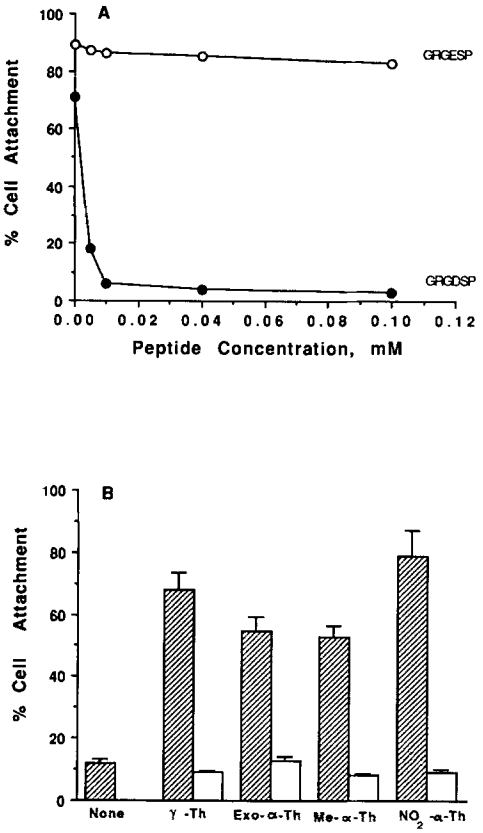

Figure 2. Effect of synthetic hexapeptides on EC attachment. (*A*) NO_2-α-thrombin. ECs were tested for attachment to NO_2-α-thrombin (20 μg/well) in the presence of increasing concentrations of either GRGDSP (●), or the control peptide GRGESP (○). The attachment of EC (2 h, 37°C) was analyzed by the methylene blue uptake assay. Cell adherence was expressed as percentage of cells seeded (1.3 × 10^5 cells/well). (*B*) Various modified thrombin analogues in the absence (□) and presence (□) of GRGDSP peptide. *γ-Th*, γ-thrombin; *Exo-α-Th*, Exo-site affinity label thrombin; *Me-α-Th*, $MeSO_2$-α-thrombin; *NO_2-α-Th*, NO_2-α-thrombin.

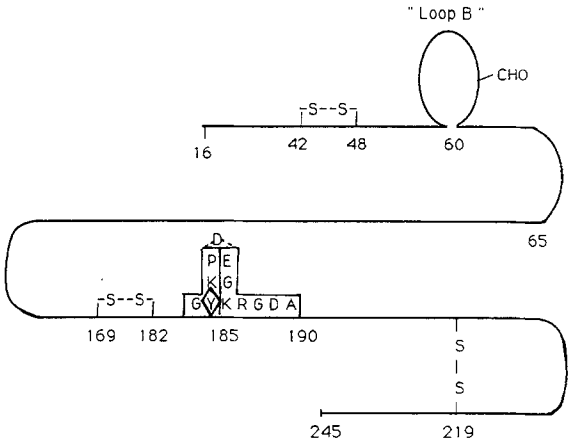

Figure 3. Schematic presentation of the human thrombin B-chain. Residues are aligned with residues 16–245 of bovine chymotrypsinogen. Individual residues are identified by the single letter code, Tyr (*Y*) in hexagons. The carbohydrate (*CHO*) attachment at Asn 60E is assumed from the bovine enzyme (26). The disulfide bonds (*S–S*) are shown above the sequence line.

Effect of Synthetic Peptides on EC Attachment to NO_2-α-thrombin

We next investigated the involvement of RGD in EC attachment to thrombin. For this purpose increasing concentrations (0.001–0.1 mM) of the synthetic hexapeptides GRGDSP or GRGESP were tested for their ability to inhibit EC attachment to NO_2-α-thrombin. While >95% inhibition was obtained in the presence of 0.01 mM GRGDSP, no significant inhibition was observed with GRGESP (Fig. 2 *A*). Similarly, GRGDSP inhibited completely the attachment of EC to various thrombin preparations (Fig. 2 *B*). These results indicate that the attachment activity of various modified thrombin preparations can be attributed to an RGD sequence in the protein. As demonstrated in Fig. 3, RGDA sequence in thrombin is located downstream at residues 187–190 of human thrombin B-chain, when residues are aligned with residues 16–245 of bovine chymotrypsin (26). Other described functional domains in thrombin, outside the proteo-

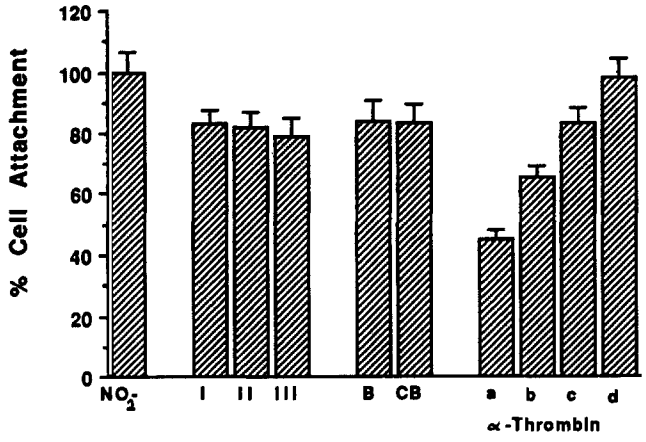

Figure 4. Effect of various thrombin fragments on EC attachment to NO_2-α-thrombin. Attachment (2 h, 37°C) of EC to NO_2-α-thrombin coated dishes in the absence (*NO_2-*) and presence of synthetic peptides (20 μg/ml) corresponding to the loop B mitogenic site (*I*, 14 amino acids; *II*, 10 amino acids; and *III*, 6 amino acids), Loop B (*B*), the chemotactic peptide CB67-120 (*CB*), and increasing concentrations of soluble α-thrombin (*a*, 20 μg/ml; *b*, 10 μg/ml; *c*, 2 μg/ml; *d*, 0.2 μg/ml). The number of firmly attached ECs was determined by the methylene blue uptake assay. Attachment in the presence of each protein is expressed as percentage of the number of cells attached to NO_2-α-thrombin alone (= 100%).

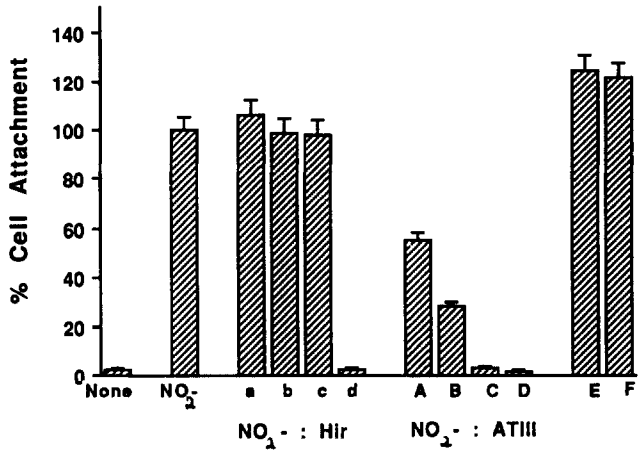

Figure 5. Effect of thrombin inhibitors on EC attachment to NO_2-α-thrombin. Dishes were coated with NO_2-α-thrombin (20 μg/ml, 0.57 nmol). Before coating, NO_2-α-thrombin was incubated with increasing amounts of hirudin or ATIII (0.57–2.85 nmol) plus heparin (0.3 U/ml), at a ratio of NO_2-α-thrombin:inhibitors of 1:1 (*a*, *A*), 1:2 (*b*, *B*), or 1:5 (*c*, *C*) for NO_2-:Hir and NO_2-:ATIII, respectively. Dishes were also coated with NO_2-α-thrombin (NO_2-; 20 μg/ml, 0.57 nmol), hirudin (*d*; 20 μg/ml, 2.85), ATIII (*D*; 171 μg/ml, 2.85 nmol), fibronectin (*E*; 30 μg/ml, 0.57 nmol), or fibronectin/ATIII (*F*; 30:171 μg/ml; 0.57:2.85 nmol). Attachment of EC was analyzed by the methylene blue uptake assay. Adhesion to NO_2-α-thrombin was regarded as 100%.

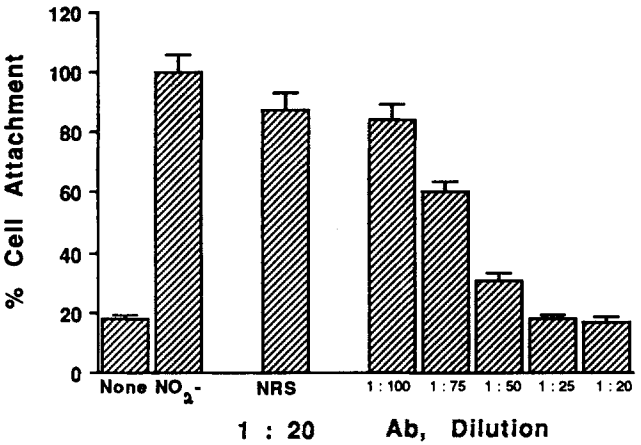

Figure 6. Effect of antiprothrombin antibodies on EC attachment to NO_2-α-thrombin. Increasing amounts of antiprothrombin antibodies or normal rabbit serum (*NRS*) were incubated (2 h, 37°C) with ECs (1.3×10^5 cells/well) in contact with NO_2-α-thrombin coated wells. Cells were also incubated in wells coated with NO_2-α-thrombin alone (*NO_2-*). Attachment of ECs was determined by the methylene blue uptake assay. 100% = adhesion to NO_2-α-thrombin alone.

lytic pocket, are located upstream to the RGDA region in distinct and separate regions. The Loop-B domain located between residues 60 and 61 within the variable region 2 (VR2) of thrombin B-chain, while the chemotactic peptide CB67-129 is located at residues 33–84 according to this numbering system. When the synthetic peptide corresponding to Loop B mitogenic site, as well as various constructs of this region were tested for their ability to compete with EC attachment to NO_2-α-thrombin, only 10–20% inhibition was observed. Similarly, the thrombin-derived chemotactic fragment CB67-129 inhibited EC attachment to thrombin by ~15%. In contrast, soluble α-thrombin inhibited the attachment of EC to NO_2-α-thrombin in a dose-dependent manner (55% inhibition at 20 μg/ml) (Fig. 4). This may indicate that the RGD domain in thrombin is only partially cryptic, and that additional neighboring sequences may assist in recognition of this RGD sequence by the appropriate cell surface receptors. The low attachment-inducing activity of native α-thrombin (Fig. 1) does not appear to be due to inactivation as a result of coating on a solid support, since all the other modified thrombin preparations retained their cell attachment activity both when present in solution (competition experiments) or when adsorbed onto plastic or glass surfaces. Moreover, plastic-bound α-thrombin exhibited other functional properties such as esterolytic activity, induction of platelet activation, and smooth muscle cell proliferation (data not shown).

Effect of Specific Thrombin Inhibitors on EC Attachment

The question arose as to whether thrombin when forming a complex with either its physiological inhibitor ATIII or with the leech-derived high affinity inhibitor hirudin, retains attachment activity. To test this possibility, equimolar amounts of thrombin and ATIII in the presence of 0.3 U/ml heparin as a catalyst were used to coat the dishes. As shown in Fig. 5, 45% inhibition of EC attachment to NO_2-α-thrombin was observed. This inhibition increased as the amount of ATIII increased, reaching 75 and 93% inhibition at a ratio of 1:2 and 1:5 thrombin to ATIII, respectively. Under these conditions the amount of surface-adsorbed thrombin was not affected by the levels of ATIII, as indicated by the similar amounts of bound ^{125}I-α-thrombin (data not shown). The specificity of this ATIII-induced inhibition of EC attachment to NO_2-α-thrombin was demonstrated by a lack of inhibition of EC adhesion to fibronectin, even at a ratio of 1:5 fibronectin/ATIII (Fig. 5). On the other hand, hirudin, the leech-derived high affinity inhibitor of thrombin, did not inhibit EC attachment to NO_2-α-thrombin even at a ratio of 1:5 thrombin/hirudin (Fig. 5).

Antiprothrombin antibodies, which also recognize thrombin, inhibited in a dose-dependent manner EC attachment to thrombin. As demonstrated in Fig. 6, complete inhibition was obtained at a 1:25 dilution of the antiserum, while there was no significant effect in the presence of preimmune serum. This inhibition further demonstrates the specificity of EC attachment to thrombin.

Effect of NO_2-α-Thrombin on EC Cytoskeletal Organization

As demonstrated in Fig. 7, NO_2-α-thrombin induced spreading of EC in a manner comparable to the subendothelial extracellular matrix. The distribution of the microfilamentous cytoskeleton and the localization of vinculin were studied by fluorescence microscopy on fixed and permeabilized EC (Fig. 8). F-actin was visualized by fluorochrome-labeled phalloidin and vinculin was identified with a specific monoclonal antibody that recognizes the mammalian form of vinculin. The extent of adhesion was evaluated by IRM, a technique that records the distance between the adhesion substratum and the cell ventral membrane. The smaller the distance the darker the signal intensity, black indicating tight adhesion. As shown in Fig. 8, upon plating of EC on NO_2-α-thrombin the cells spread and organized a network of thick microfilamentous bundles. Formation of stress fibers occurred with appearance of vinculin streaks at their endings in correspondence with focal contacts. These cells also showed multiple IRM black streaks that corresponded to the endings of stress fibers and distinct arrowhead-shaped vinculin speckles. Cell spreading and cytoskeletal organization on NO_2-α-thrombin was comparable to that observed upon seeding of EC on a vitronectin substratum (15). Immunofluorescence staining using anti-α_v and anti-β_3 antibodies (Fig. 9) gave a peculiar pattern of oval and arrowhead-shaped spots usually located at stress fiber endings. The amount of β_3 clusters was, however, reduced in comparison to EC adhering to vitronectin (Fig. 9). A comparable distribution was observed in studies with HUVEC using anti-α_v (LM142) mAb (Fig. 9), or an anti-$\alpha_v\beta_3$ complex mAb (LM609), or anti-β_3 monoclonal and polyclonal antibodies (data not shown). On BAEC only the polyclonal anti-β_3 antibodies gave a good staining at adhesion plaques, while staining with the monoclonal antibodies used in this study was less easily detectable, possibly due to lack of cross-reactivity.

Anti-$\alpha_v\beta_3$ antibodies (LM609) blocked EC adhesion to NO_2-α-thrombin (Fig. 10) and subsequent cell spreading (not shown). In contrast, the mAb BIE5 and an anti-$\alpha_5\beta_1$ goat antiserum were ineffective at concentrations that markedly inhibited EC adhesion to fibronectin. These results indicate that the $\alpha_v\beta_3$ integrin is involved in EC adhesion to NO_2-α-thrombin.

We investigated the possible involvement of ECM proteins synthesized and secreted by EC during the process of cell adhesion to NO_2-α-thrombin. For this purpose EC were treated with either emetine to block protein synthesis, or with monensin to prevent glycoprotein secretion. No significant effect on the extent of cell adhesion was observed with both types of treatments (data not shown).

Attachment of EC to Native α-Thrombin

To explore the possibility that under certain conditions (i.e., limited autoproteolysis) the RGD region of native α-thrombin, can be exposed and function in promoting cell adhesion, the enzyme was preincubated at 37°C before coating of plastic surfaces. Fig. 11 demonstrated that α-thrombin that was first incubated at 37°C was a potent inducer of EC attachment as compared to lack of such an activity in the absence of preincubation. This effect was rather rapid since a significant attachment activity was observed even after a 15-min preincubation of α-thrombin at 37°C. When the protease inhibitor TLCK was present during preincubation of thrombin at 37°C, the ability of thrombin to induce EC attachment subsequent to coating of plastic surfaces was reduced by >65% (data not shown). These data suggests that preincuba-

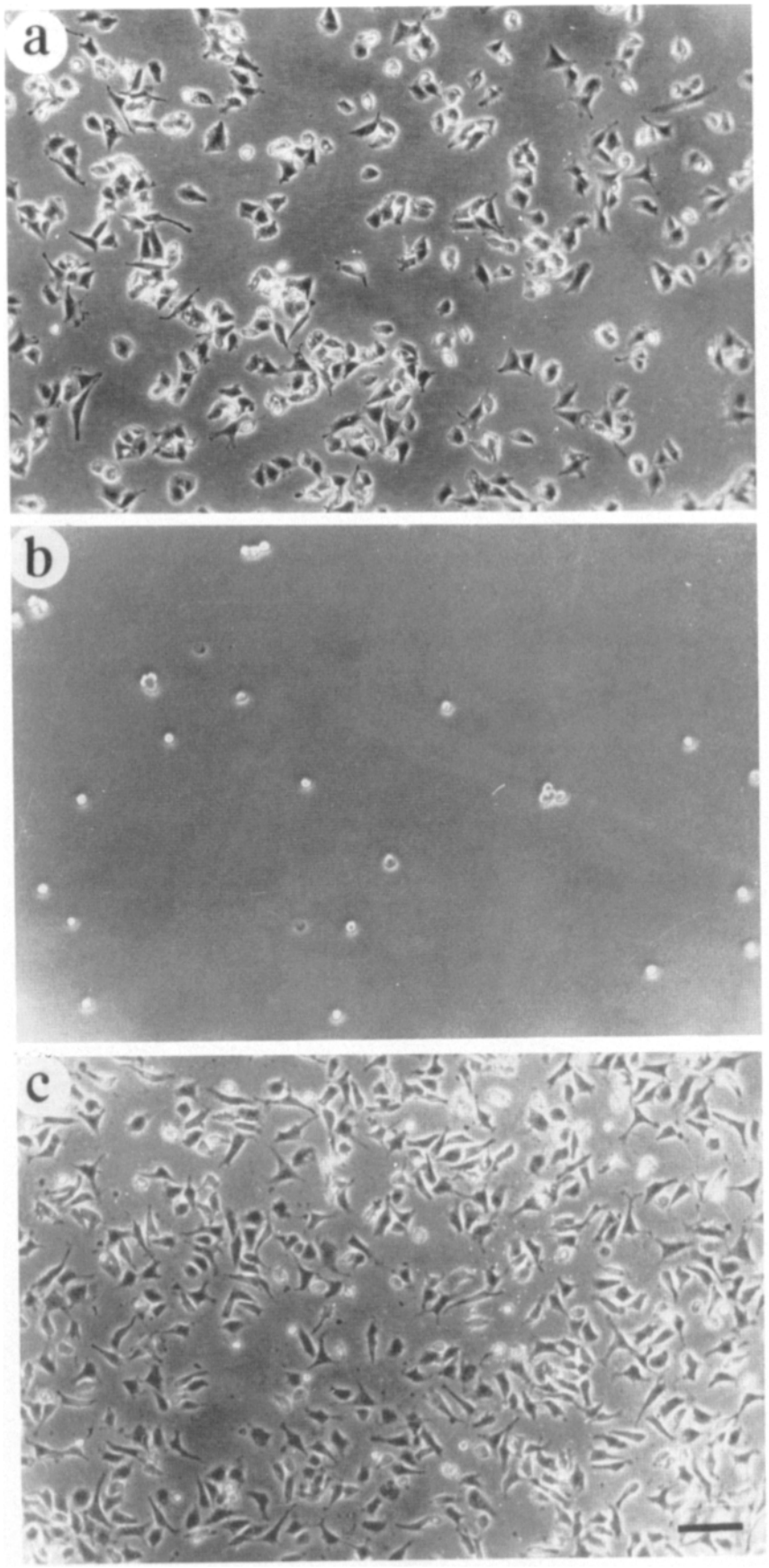

Figure 7. Attachment of endothelial cells to NO_2-α-thrombin. Culture dishes were coated with 20 μg/ml of (*a*) NO_2-α-thrombin or (*b*) BSA. Dishes were also coated with (*c*) naturally produced subendothelial ECM. Phase micrographs were taken after 2-h incubation at 37°C with the cells. Bar, 100 μm.

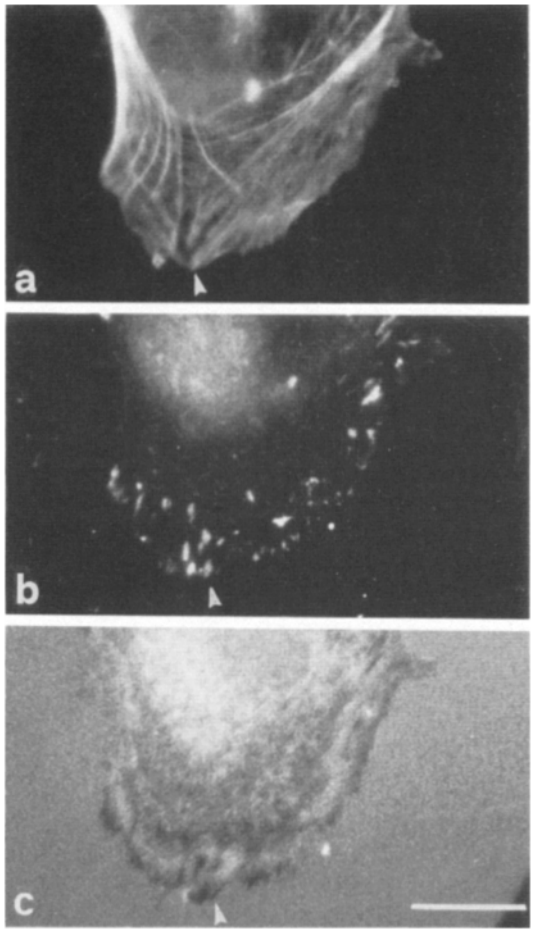

Figure 8. HUVECs attach, spread, and organize focal contacts when plated on NO_2-α-thrombin. Cell attachment was associated with (*a*) stress fiber formation, and (*b*) vinculin organization at stress fiber endings. These endings were found to correspond to dark spots in interference reflection microscopy (*c*). The arrowheads in *a*, *b*, and *c* correspond to the position of a single focal contact. Bar, 10 μm.

tion at 37°C and the associated autoproteolysis of the thrombin molecule may result in surface exposure of the RGD domain in thrombin, which may hence mediate EC adhesion after adsorbance to the tissue culture plastic.

Discussion

Thrombin, the central bioregulatory enzyme in hemostasis, is actively engaged in governing thrombus formation. During the initial phases of the hemostatic response after vessel injury, thrombin is generated to produce a fibrin network, promoting further fibrin dependent aggregation of platelets adherent to the exposed subendothelium (7, 33, 35, 41). These processes contribute to the formation of thrombus that initially seals the vessel to prevent excessive blood loss. During subsequent wound healing, after vessel injury, ECs initiate local repair mechanisms involving cell attachment, migration, and proliferation to renew the damaged vessel. The adhesive phenotype of endothelial cells appears to be a critical factor regulating this repair process. Therefore, to understand the molecular events involved in thrombus formation and wound healing, it is necessary to delineate structural interactions between cells and molecules participating in these processes.

In this report, we present data showing that the RGD sequence within thrombin promotes EC adhesion, spreading, and cytoskeletal reorganization. Modifications of the thrombin molecule either at the procoagulant or catalytic site gave rise to species of thrombin that were highly adhesive for endothelial cells. This was found for thrombin that was catalytically blocked by $MeSO_2$ at the active site serine or by the fibrinopeptide exosite affinity label. ECs attached also to γ-thrombin, exhibiting no procoagulant activity and a low proteolytic function. Furthermore, nitration of tyrosine residues which yielded a reduced procoagulant activity, resulted in a maximal induction of EC attachment. Indeed, amino acid analysis shows the presence of a tyrosine residue located at position 185 of thrombin B-chain, preceding the only RGDA sequence in the protein (residues 186–189). This RGD sequence in thrombin is apparently hindered, located inwards towards the core of the enzyme and therefore is not exposed in the native molecule, which exhibits a very low attachment activity. In support of these findings are recent crystallographic studies indicating that the RGD domain is not accessible on the surface of the thrombin molecule (8). Upon specific modifications, however, this region may be exposed, for example, by nitration of the tyrosine residue adjacent to the RGD sequence. This tyrosine (Tyr-185) apparently plays a significant role in altering the conformation of α-thrombin, resulting in exposure of RGD sequence at an exosite domain. The degree of nitration (eight or three nitrotyrosines per mole) did not affect significantly the attachment-promoting activity of the modified thrombin (data not shown).

EC attachment was completely abolished in the presence of ATIII, forming a complex with NO_2-α-thrombin, suggesting that ATIII masks the RGD region in thrombin. In contrast, hirudin, a leech-derived protein (M_r ~7,000) with high affinity to thrombin, did not inhibit its attachment promoting activity. Hirudin has a negatively charged tail (1, 19, 34), thought to bind to a region neighboring the catalytic site located upstream to the RGD sequence at the frontal side of thrombin. The RGD domain is therefore an unlikely candidate for interaction with hirudin (21). Complexes of NO_2-α-thrombin and hirudin served as potent attachment substrates for EC, in correlation with the described thrombin-hirudin interaction regions (thrombin chemotactic and mitogenic domains) and the distinct RGD domain responsible for EC attachment. When anti-prothrombin antibodies were present during the adhesion assay, cell attachment was completely blocked, further reflecting the specific nature of EC-thrombin interactions.

EC spreading on NO_2-α-thrombin was followed by actin microfilament assembly, and appearance of interference reflective microscopy black streaks and vinculin speckles at stress fiber endings. Qualitatively, this type of cellular responses were comparable to those observed after seeding the cells on a series of characteristic matrix proteins such as vitronectin, fibronectin, or von Willebrand factor (13, 15, 17, 18). This phenomenon is of particular relevance because it indicates that the plasma membrane has interacted with the substratum via specific receptors and that this interaction has triggered a cascade of events leading to the organization of

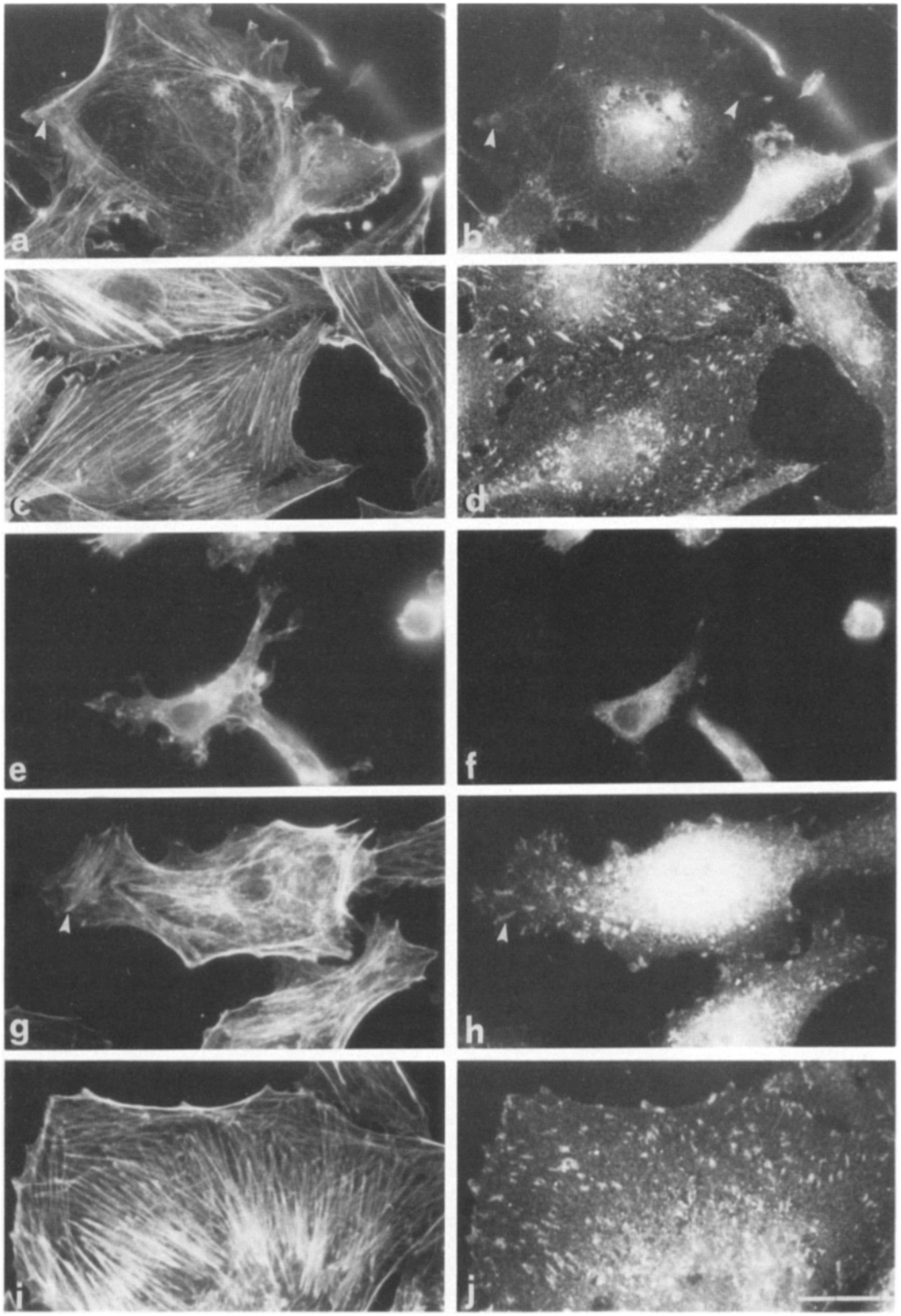

Figure 9. Distribution of α_v receptors, β_3 receptors, and F-actin HUVECs (*a–f*) and BAECs (*g–j*) were incubated (1.3×10^5 cells/well) for 4 h on coverslips coated with vitronectin (*c*, *d*, *i*, and *j*), NO_2-α-thrombin (*a*, *b*, *g*, and *h*), or thrombin (*e* and *f*). The cells were permeabilized and stained with anti-α_vmAb (LM142) (*b*, *d*, and *f*), rabbit anti-GpIIIa antiserum (MAR-2) (*h* and *j*), or F-PHD (*a*, *c*, *e*, *g*, and *i*). Plating of HUVECs on NO_2-α-thrombin supports spreading and stress fiber formation to a lesser extent than on vitronectin (compare *a* and *c* and *b* and *d*, respectively); α_v clusters were fewer and more weakly stained, see arrowheads (*b* and *d*). In HUVECs the organization of β_3 was comparable to that of α_v (not shown). Plating on α-thrombin did not result in stress fiber formation or integrin receptor organization (*e* and *f*). BAECs plated on NO_2-α-thrombin (*g* and *h*) adhered but exhibited a bit less spreading and stress fiber formation than on vitronectin (*i* and *j*). Such reduced flattening was reflected in a less elaborate pattern of β_3 receptor clustering and focal contact formation on NO_2-α-thrombin than on vitronectin (compare *h* and *j*). Bar, 10 μm.

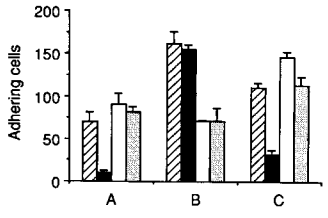

Figure 10. Effect of integrin receptor antibodies on HUVEC adhesion to different substrata. HUVEC were seeded on NO_2-α-thrombin (100 μg/ml; *A*); fibronectin (3 μg/ml; *B*); or vitronectin (3 μg/ml; *C*). Before the adhesion assay HUVEC were incubated for 30 min at 37°C with: buffer (▨); $\alpha_v\beta_3$ antibody, LM609 mAb; (■); α_5 antibody, β1E5 mAb, (□); $\alpha_5\beta_1$ goat antiserum (▩). When goat nonimmune serum or mAb 10E3 were used, no change in cell adhesion was observed. Adhesion to BSA (1% in coating) was 7 ± 1. Data are means ± SEM of five replicates from one experiment out of four performed with comparable results.

adhesion plaques in which vinculin and other proteins are associated. It is therefore likely that the effect of NO_2-α-thrombin on cell spreading and cytoskeletal organization is mediated by a specific recognition mechanism.

Inhibition studies revealed that an anti-$\alpha_v\beta_3$ mAb was able to block adhesion of EC to NO_2-α-thrombin, while anti-$\alpha_5\beta_1$ antibodies were ineffective. Vitronectin receptor ($\alpha_v\beta_3$) was also found to be clustered in focal contacts upon EC adhesion to NO_2-α-thrombin. Altogether these data strongly suggest that $\alpha_v\beta_3$ plays a leading role in mediating EC interaction with NO_2-α-thrombin. The vitronectin receptor (39) is very promiscuous and recognizes, besides vitronectin, also other substrata including von Willebrand factor, and fibrinogen (11, 32). The data reported here provide evidence that this receptor binds modified thrombin as well, thus identifying a novel ligand for this integrin. Seeding of EC on NO_2-α-thrombin resulted in deposition of fibronectin that could influence cell adhesion. However, blocking endogenous matrix formation by treatment with inhibitors of protein synthesis and secretion (14, 40), had no effect on EC adhesion and spreading onto NO_2-α-thrombin (data not shown). This indicates that NO_2-α-thrombin itself could directly induce EC adhesion and cytoskeletal organization.

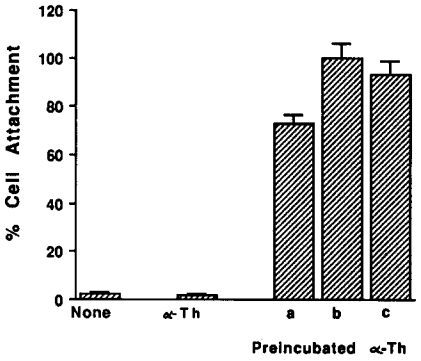

Figure 11. Adhesion of EC to native α-thrombin. Culture dishes were coated with 20 μg/ml of α-thrombin (α*Th*), or with 20 μg/ml of thrombin that was first incubated in solution at 37°C for 60 min (*a*); 30 min (*b*); or 15 min (*c*). Cells were also incubated with dishes coated with BSA alone (*None*). The number of attached ECs was determined by the methylene blue uptake assay. 100% attachment represents the number of cells attached to thrombin that was preincubated at 37°C for 60 min. This value was similar to that obtained on NO_2-α-thrombin coated dishes under the same conditions.

The biological relevance of the adhesive properties of modified thrombin molecules remains to be fully elucidated. The native enzyme has very little adhesive properties, despite having an RGD domain. However, it may still compete for adhesion to modified NO_2-α-thrombin (Fig. 4) and with other adhesive proteins, for example fibronectin (data not shown). The fact that thrombin molecule is composed of various functional domains some of which are exosite exposed regions and others like the RGD domain, are hindered, is intriguing. We have demonstrated that preincubation of α-thrombin at 37°C for a period of 15–60 min before coating of the plastic surface resulted in potent adhesive properties (Fig. 11). This could in part be due to autoproteolysis of the enzyme (as was indeed indicated by the inhibitory effect of TLCK) and/or to other structural modifications of the molecule occurring during incubation at 37°C. We postulate that the RGD site can be surface exposed to response to specific modifications and signals which may be triggered under stress or pathological conditions. In would healing and inflammatory processes, extensive recruitment of leukocytes takes place. Moreover, it has been demonstrated that thrombin that is ubiquitous to vascular injury, is a potent chemotaxin for mononuclear phagocytes (3). Therefore, we suggest that during tissue repair when excess of mononuclear-phagocytic cells (highly rich in proteolytic enzymes) are present, these cells may act on thrombin in a manner that will modify the molecule and expose the RGD domain for direct participation in cellular attachment. The effect of various proteolytic enzymes (i.e., elastase, plasmin, cathepsin G, etc.) on thrombin attachment activity is under a current investigation.

In conclusion, we present evidence that certain modifications of thrombin may result in "pointing out" of its RGD region that may then be actively engaged in supporting EC adhesion and cytoskeletal reorganization. We suggest that thrombin, in addition to playing a pivotal role in hemostasis, participates in repair mechanisms and maintenance of the integrity of the internal vessel lining by acting as a matrix protein for EC. Our data on the involvement of the $\alpha_v\beta_3$ integrin in promoting EC adhesion to modified thrombin identify a new binding site for this molecule on EC.

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