Thrombin induces endothelial cell growth via both a proteolytic and a non-proteolytic pathway

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Binding of ^{125}I-thrombin to human umbilical vein endothelial cells (HUVECs) was specifically displaced by the synthetic tetradecapeptide SFLLRNPNDKYEPF, named thrombin receptor agonist peptide (TRAP), which has recently been described as a peptide mimicking the new N-terminus created by cleavage of the thrombin receptor, and F-14, a tetradecapeptide representing residues 365–378 of the human α-thrombin B chain. Binding of ^{125}I-TRAP to HUVECs was time-dependent, reversible and saturable, showing high affinity ($K_D = 1.5 \pm 0.4$ μM) and high binding capacity ($B_{max.} = 7.1 \pm 0.6 \times 10^6$ sites/cell) ($n = 3$). Unlabelled thrombin and TRAP competitively and selectively inhibited the specific binding of ^{125}I-TRAP with IC_{50} values of 5.8 ± 0.7 nM and 2.8 ± 0.4 μM respectively, whereas F-14 remained ineffective at displacing ^{125}I-TRAP from its binding sites, suggesting the presence of at least two different types of thrombin-binding sites on HUVECs. TRAP was a potent mitogen for HUVECs in culture. Both TRAP and α-thrombin stimulated the proliferation of HUVECs with half-maximum mitogenic responses between 1 and 10 nM. F-14 also promoted HUVEC growth. The mitogenic effects of F-14 and TRAP were additive. Nα-(2-Naphthylsulphonylglycyl)-DL-*p*-amidinophenylalanylpiperidine (NAPAP) and hirudin (two specific inhibitors of the enzymic activity of thrombin) specifically inhibited thrombin-induced HUVEC growth (IC_{50} values 400 ± 60 and 52 ± 8 nM respectively) but remained without effect on the mitogenic effect of TRAP or F-14. This demonstrated that the mitogenic effect of α-thrombin for HUVECs was intimately linked to its esterolytic activity but also showed that thrombin can stimulate HUVEC growth via another non-enzymic pathway. This hypothesis was further reinforced by the fact that F-14-induced proliferation of HUVECs remained unaltered by two antibodies directed against TRAP or the cleavage site on the extracellular portion of the thrombin receptor, which both strongly reduced thrombin-induced proliferation of HUVECs. Thrombin-, TRAP- or F-14-induced HUVEC proliferation was strongly inhibited by a neutralizing monoclonal antibody directed against basic fibroblast growth factor (bFGF), suggesting that thrombin regulates the autocrine release of bFGF in HUVECs.

INTRODUCTION

The multifunctional serine protease α-thrombin, generated at sites of vascular injury, has central functions in haemostasis but also promotes a wide range of cellular responses [1]. It is a potent activator of platelets [2] and presents a variety of functions upon inflammatory and vascular cells. Thrombin is chemotactic for monocytes and mitogenic for lymphocytes [3,4]. In cultured endothelial cells, thrombin induces a large variety of cellular responses, among which are prostacyclin and plasminogen-activator-inhibitor secretion [5–7], growth-related signals such as activation of phospholipase C with generation of inositol trisphosphate and increase in intracellular Ca^{2+} concentration, stimulation of Na^+/H^+ exchange and intracellular alkalinization or induction of the proto-oncogenes c-*fos* and c-*cis* [8–10]. Thrombin has a direct mitogenic effect on rat neonatal and bovine aortic smooth muscle cells, quiescent fibroblasts and vascular endothelial cells [8,11–16]. The enzymic activity of thrombin seems to be necessary for its mitogenic effect on smooth muscle cells [16,17]. Molecular cloning of a functional thrombin receptor on platelets and vascular endothelial cells revealed a novel proteolytic mechanism of receptor activation [18]. These studies described a new receptor activation mechanism in which α-thrombin cleaved its receptor's N-terminal extension site to create a new N-terminus that functioned as a tethered ligand and activated the receptor. A synthetic peptide of 14 residues (SFLLRNPNDKYEPF) (hereby named thrombin receptor agonist peptide, TRAP), corresponding to the new N-terminal portion of the receptor, was shown to activate the cloned thrombin receptor and to induce platelet activation, Ca^{2+} fluxes in endothelial cells and platelets, or smooth muscle cell proliferation [16–18]. Since this ligand can only be generated by the catalytic action of the enzyme, part of our work consisted of studying the effect of this synthetic agonist peptide on the growth of human umbilical vein endothelial cells (HUVECs) in culture.

Recently, it has been demonstrated that two TRAP-dependent and -independent pathways were involved in thrombin-induced enhancement of vascular endothelium permeability [19], suggesting that dual signals could also be involved in the mechanism of thrombin-induced mitogenesis. We therefore investigated the possibility that thrombin might induce mitogenicity in endothelial cells via both thrombin cell receptor-dependent and -independent pathways. In doing so, we compared the mitogenic effect of α-thrombin, TRAP and F-14, a synthetic tetradecapeptide representing the 'loop B' region of the human α-thrombin (residues 365–378) which has been shown to exhibit mitogenic properties on J 774 cells, a murine macrophage-like tumour cell line [20].

We also determined to what extent the mitogenic effect of thrombin, as well as the synthetic peptides, might be mediated by

HUVECs, human umbilical vein endothelial cells; TRAP, thrombin receptor agonist peptide; NAPAP, Nα-(2-naphthylsulphonylglycyl)-DL-*p*-amidinophenylalanylpiperidine; bFGF, basic fibroblast growth factor; FCS, fetal-calf serum; mAb, monoclonal antibody; PDGF, platelet-derived growth factor; ECGF, endothelial cell growth-promoting factor; PPACK, D-Phe-Pro-Arg-chloromethane; DFP, di-isopropylfluorophosphate.

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basic fibroblast growth factor (bFGF), as has already been shown for the mitogenic effect of thrombin on vascular smooth muscle cells [21].

MATERIALS AND METHODS

Chemicals

F12-Ham's medium, fetal calf serum (FCS), glutamine, streptomycin and penicillin were purchased from Boehringer–Mannheim (France). Standard heparin (from pig intestinal mucosa, 168 i.u./mg) and ECGF were from Sigma Chemical Co. (France). *N*α-(2-naphthylsulphonylglycyl)-DL-*p*-amidinophenylalanylpiperidine (NAPAP), recombinant hirudin (rHV2-Lys-47 variant) and the synthetic peptides TRAP (SFLLRNPNDKYEPF), F-14 (LLYPPWNKNFTEND) and F-10 to F-13 were from Sanofi Recherche (Toulouse, France). Human α-thrombin (3000 NIH units/mg) was purchased from Centre regional de transfusion sanguine (Strasbourg, France). Monoclonal mouse anti-(bovine bFGF) (mAb-bFGF) and monoclonal mouse anti-[human platelet-derived growth factor BB (PDGF-BB)] antibodies (mAb-PDGF) were obtained from UBI (Lake Placid, NY, U. S. A.). Neutralizing antisera directed against TRAP and TRC (KATNATLDPR), a decapeptide adjacent to the cleavage site on the extracellular portion of the thrombin receptor, were raised in a goat as described previously [22].

Culture of HUVECs

HUVECs were isolated and cultured as described [23] in 75 cm^2 culture flasks in F12-Ham's medium supplemented with 10% FCS, penicillin (100 i.u./ml), streptomycin (100 μg/ml), glutamine (2 mM), endothelial cell growth-promoting factor (ECGF; 30 μg/ml) and heparin (100 μg/ml). Cells were routinely used from the first to the sixth passage.

Cell proliferation assays

Cells were plated sparsely (1×10^4 cells/well) in 24-well cluster plates (Nunc, Denmark) in F12-Ham's medium containing 0.5% FCS. After 1 day, fresh medium was added to the remaining dishes (F12-Ham's medium containing 0.5% FCS, α-thrombin or the synthetic peptides and the different concentrations of the drugs to be tested). For growth rate determinations, after 5 days in culture, cells were detached from triplicate wells by trypsin treatment (0.05% trypsin containing 0.02% EDTA) and counted in a coulter counter (Coultronics, France). IC_{50} values were calculated on the basis of the linear regression lines established for each compound tested.

Binding of ^{125}I-thrombin and ^{125}I-TRAP to HUVECs

Thrombin and TRAP were radiolabelled with Na^{125}I using Iodo-Gen beads (Pierce Chemical Co., Interchim, Montluçon, France) for 7 min at 22 °C. Unbound iodine was removed by passing the labelled compounds over a PD-10 column (Pharmacia LKB Biotechnology, Saint Quentin en Yvelines, France) pre-equilibrated with PBS containing 0.01% Tween 80. Thrombin was labelled to a specific radioactivity of 2–4 μCi/μg and retained about 80% of its amidolytic activity. Experiments studying the specific binding of ^{125}I-thrombin and ^{125}I-TRAP to HUVECs were performed on adherent cells cultured in 24-well cluster plates. Culture medium was removed and confluent cells (approx. 5×10^5 cells/well) were washed twice with 1 ml of ice-cold PBS. Incubations were carried out in a total 0.2 ml volume of PBS which contained 2% (w/v) BSA and ^{125}I-thrombin (50 nM) or ^{125}I-TRAP (1 μM). Triplicate incubations were carried out at 15 °C for 60 min and were terminated by the addition of 1 ml of ice-cold assay buffer. Cells were then rapidly washed twice with 1.5 ml of ice-cold incubation buffer and the radioactivity measured after digestion of the cell monolayer with 0.5 ml of an aqueous 0.3% (w/v) Triton X-100 solution. Non-specific binding was defined as the total binding measured in the presence of excess unlabelled thrombin or TRAP (1 and 10 μM respectively) and specific binding was defined as the difference between total binding and non-specific binding. The apparent dissociation constants (K_D) and the maximal number of binding sites ($B_{max.}$) were calculated by using a Scatchard representation of the experimental data using a non-linear regression program (Kinetic, Biosoft, Cambridge, U.K.).

RESULTS AND DISCUSSION

Binding of ^{125}I-thrombin and ^{125}I-TRAP to HUVECs

^{125}I-thrombin bound to a single class of high-affinity binding sites on HUVECs, the dissociation binding constant (K_D) and the maximal binding capacity ($B_{max.}$) values being of 6.3 ± 0.9 nM and $(8.9 \pm 0.9) \times 10^6$ sites/cell ($n = 3$) respectively. This specific binding was inhibited in a monophasic manner by unlabelled thrombin, F-14 or TRAP with IC_{50} values (concentrations which inhibited 50% of the specific binding of ^{125}I-thrombin) of 2.4 ± 0.8 nM, 30 ± 7.4 μM and 1.8 ± 0.3 μM respectively ($n = 3$) (Figure 1a). Interestingly, neither TRAP nor F-14 were able to totally displace ^{125}I-thrombin from its binding sites on HUVECs. It is noteworthy that, under our experimental conditions, inhibition of thrombin binding was not due to downregulation of the receptor following activation by TRAP or thrombin. Indeed, we took great care in our binding experiments not to be influenced by the proteolytic action of thrombin and, for this reason, all the experiments were performed at 15 °C. TRAP inhibited ^{125}I-thrombin binding in a competitive manner, as demonstrated by a Scatchard representation of the data (results not shown) and by the calculation of the Hill coefficient (1.07 ± 0.11, $n = 6$). In a similar manner, prolonged incubation (1 h at 15 °C) with TRAP (10 μM) and extensive washing prior to ^{125}I-thrombin binding did not result in a modification of the binding characteristics of the ligand, therefore suggesting that the inhibitory effect of TRAP was not due to the uptake of the thrombin receptor following activation.

Specific binding of ^{125}I-TRAP to HUVECs at 15 °C was time-dependent and reached an equilibrium within 45 min of incubation (results not shown). Binding of ^{125}I-TRAP to HUVECs was dose-dependent and the specific binding, defined as the total amount of ^{125}I-TRAP bound minus the non-specific binding, was saturable, reaching a maximum around 5 μM. The non-linear regression analysis and the Scatchard analysis revealed the presence of one class of binding sites exhibiting high affinity with a K_D value of 1.5 ± 0.4 μM ($n = 3$) and a $B_{max.}$ value of $(7.1 \pm 0.6) \times 10^6$ sites/cell ($n = 3$). As shown in Figure 1(b), unlabelled thrombin and TRAP displaced in a dose-dependent manner ^{125}I-TRAP specifically bound to its receptor sites on HUVECs. The concentrations required to inhibit 50% of the specific binding (IC_{50}) were 5.8 ± 0.7 nM and 2.8 ± 0.4 μM ($n = 3$) for thrombin and TRAP respectively. F-14 had no effect on ^{125}I-TRAP binding to HUVECs up to a concentration of 10 μM (Figure 1b). This observation therefore indicates that, although interfering with ^{125}I-thrombin binding to HUVECs, F-14 acts on different binding sites to TRAP.

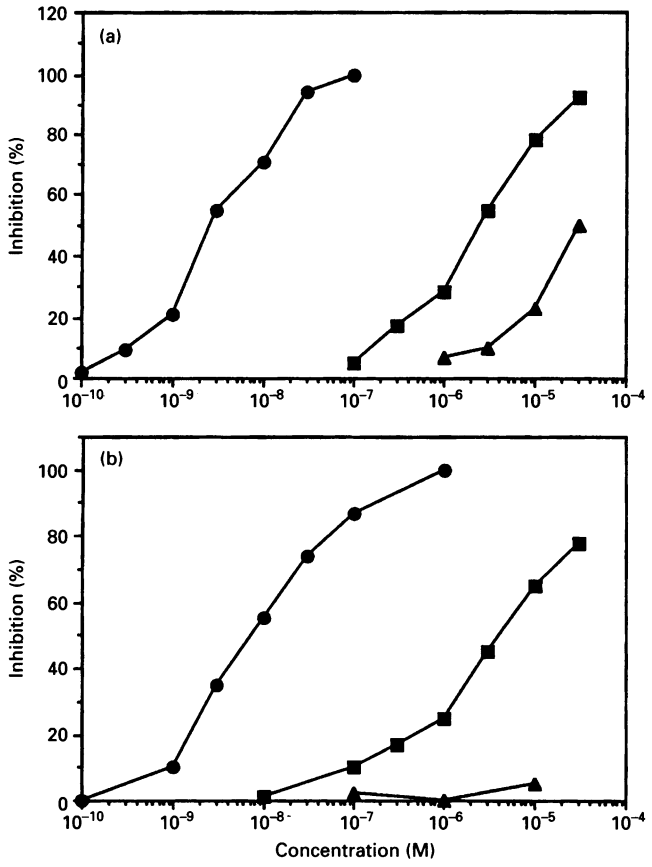

Figure 1 Binding of ^{125}I-thrombin and ^{125}I-TRAP to HUVECs

HUVECs (5×10^5 cells/well) were incubated with ^{125}I-thrombin (50 nM) (**a**) or ^{125}I-TRAP (1 μM) (**b**) for 60 min at 15 °C with increasing concentrations of thrombin (●), TRAP (■) or F-14 (▲). Binding was determined as described in the Materials and methods section. Each data point is the average of results from at least three independent determinations performed in triplicate.

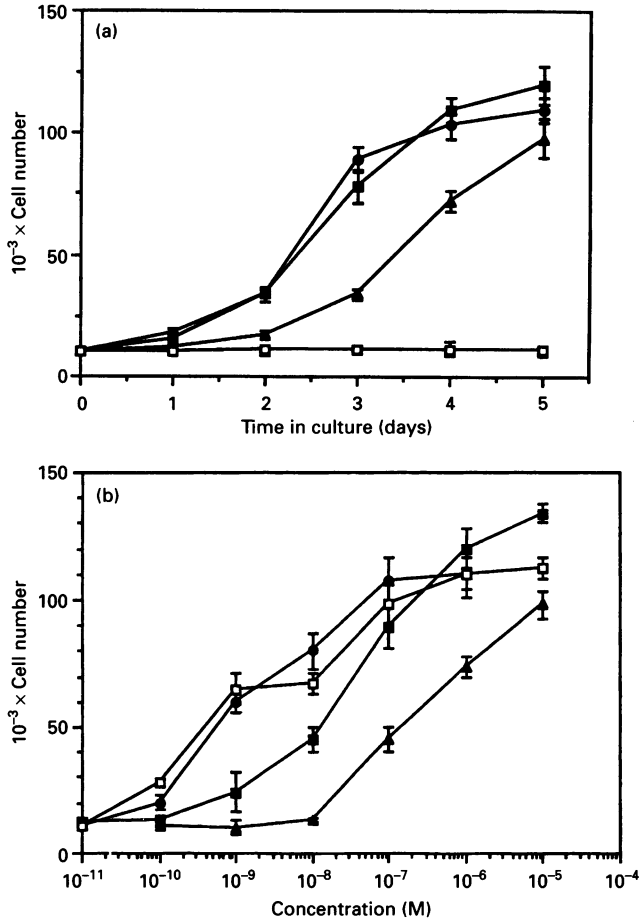

Figure 2 HUVEC growth in the presence of thrombin, TRAP or F-14

HUVECs were seeded (1×10^4 cells/well) in culture medium containing 0.5% FCS. For time-course studies (**a**), saline (□), human α-thrombin (1 μM) (●), TRAP (SFLLRNPNDKYEPF; 1 μM) (■) and F-14 (LLYPPWNKNFTEND; 10 μM) (▲) were added to the dishes. After the indicated periods of time, cells were detached from triplicate wells by trypsin treatment (0.05% trypsin/0.02% EDTA) and counted in a coulter counter. For dose–response studies (**b**), HUVECs were grown in culture medium containing 0.5% FCS with the indicated concentrations of α-thrombin (●), TRAP (■), F-14 (▲) or TRAP + F-14 (equimolar mixture) (□). After 5 days in culture, cells were trypsinized and counted. Data are reported as mean cell density ± S.D. ($n = 9$).

Effect of α-thrombin and TRAP on the proliferation of HUVECs in culture

Thrombin is a serine protease that plays a central role in initiating procoagulant events, but its involvement in the wound healing process is attracting increasing interest and it is now emerging as an important growth factor for various cell types of the vascular wall, including endothelial cells [8,11–17]. Our results confirmed these data, indicating that native α-thrombin stimulated in a time- and dose-dependent manner the growth of HUVECs *in vitro* (Figure 2). Over a period of 1 to 5 days, thrombin induced a 5–6-fold increase in cell number (Figure 2a). The concentration of α-thrombin required to obtain optimal cell proliferation ($A_{max.}$) was 0.1 μM with a half-maximal response (ED_{50}) at 4 nM (Figure 2b). This observation is consistent with already published results on endothelial cells and on other cell types [8,11–17]. TRAP, a synthetic peptide recently described to mimic the new N-terminus created by thrombin after cleavage of its receptor on platelets and endothelial cells, exhibited a significant dose-dependent mitogenic effect for HUVECs when tested at concentrations ranging from 0.01 nM to 10 μM (Figure 2b). The ED_{50} of TRAP was about 30-fold below that observed for native α-thrombin. Since this peptide has already been shown by Vu et al. [18] to mimic the new N-terminus created by cleavage of the thrombin receptor, our results show that enzymic activity of α-thrombin is necessary for its mitogenic effect on HUVECs. However, Bar-Shavit et al. showed that the thrombin molecule contains a sequence which behaves as a growth factor distinct from the enzyme active centre [20]. We therefore evaluated the mitogenic activity of a thrombin-derived chemotactic fragment, the tetradecapeptide named F-14. This peptide, representing residues 365–378 of the human B chain sequence, stimulated HUVEC growth over a concentration range of 10 nM to 10 μM, with optimal stimulation occurring at 10 μM (Figure 2b). This effect was specific for F-14, since a mismatch tetradecapeptide was unable to support HUVEC growth when tested at the concentration of 10 μM (Table 1). To determine whether the mitogenic effect of F-14 might be expressed by smaller peptide fragments, additional peptides (from 10-mer to 14-mer) were tested for their ability to induce HUVEC growth. Under our experimental conditions, the critical size required to exhibit full mitogenic activity was a 12-mer peptide, whereas smaller fragments (11-mer and 10-mer) were almost totally devoid of activity (Table 1). Remarkably, the 12-mer (thrombin 363–378) corre-

Table 1 Mitogenic effect of various thrombin-derived peptides

HUVECs (1×10^4 cells/well) were allowed to grow in the presence of culture medium + 0.5% FCS supplemented with thrombin (1 μM) and the various peptides (10 μM). Cells were counted after 5 days. Data are expressed as mean cell number ± SD ($n = 9$).

Peptides	Structure	Number of HUVECs/well ($\times 10^3$)
Saline	–	11 ± 0.2
Thrombin	–	110 ± 0.6
14-mer	LLYPPWNKNFTEND	98 ± 7.9
Mismatch 14-mer	LLYPPWNKNFTEDN	13 ± 0.9
13-mer	LYPPWNKNFTEND	103 ± 4.3
12-mer	YPPWNKNFTEND	90 ± 9.2
11-mer	PPWNKNFTEND	21 ± 1.4
10-mer	PWNKNFTEND	14 ± 2.7

sponded to the precise limits of the loop-B thrombin B chain insertion sequence [24]. Therefore, the loop-B region in the thrombin molecule appears to be responsible, at least in part, for the non-esterolytic growth factor activity exhibited by thrombin. When added simultaneously, F-14 and TRAP showed an additive mitogenic effect, the global activity being highly similar to what was obtained with α-thrombin (Figure 2b). These results show that thrombin can stimulate HUVEC growth via two different pathways, one being closely linked to its catalytic activity, the other mediated by a specific sequence located within loop B of the thrombin B chain, separate and distinct from its active centre and located at the surface of the thrombin molecule.

The specificity of thrombin-induced proliferation was further demonstrated by growing HUVECs in the presence of two selective inhibitors, NAPAP and hirudin. These direct thrombin inhibitors did not affect the mitogenic effect of ECGF/heparin, TRAP or F-14 but almost totally blocked α-thrombin-induced proliferation of HUVECs with IC_{50} values of 0.41 ± 0.06 μM and 52 ± 8 nM respectively ($n = 3$) (Figure 3). It is noteworthy that even at the highest concentration tested, both inhibitors did not totally abrogate HUVEC proliferation, a 20–30% residual mitogenic activity of thrombin remaining unaffected by 1 μM hirudin or 10 μM NAPAP, whereas at these concentrations both compounds exhibited 100% inhibition of the amidolytic activity of α-thrombin (results not shown). In order to ascertain such observations, the effect of D-Phe-Pro-Arg-chloromethane (PPACK) and di-isopropylfluorophosphate (DFP), both irreversible inhibitors of thrombin, was determined. PPACK and DFP, added in the incubation medium simultaneously with thrombin or after a 1-h preincubation followed by elimination of the unbound inhibitor by exclusion chromatography, showed results similar to those described with hirudin and NAPAP (results not shown), whereas, under these experimental conditions, no residual catalytic activity of thrombin was measured. These observations therefore confirm that partial inhibition of the mitogenic effect of thrombin by the various inhibitors was not due to reversal of the inhibitory effect with time. Similarly, co-incubation of PPACK–thrombin with thrombin plus hirudin did not result in an additional inhibitory effect, showing a 76% inhibition of the mitogenic effect of thrombin (versus 81% inhibition in the absence of PPACK–thrombin, with thrombin plus hirudin alone). These observations therefore confirm that α-thrombin functions as a potent mitogen towards HUVECs through both its catalytic activity and another non-esterolytic-dependent pathway.

To establish whether this non-enzymic activity of thrombin might be due to a specific interaction with the known thrombin receptor, antibodies were raised against TRAP (mAb-TRAP) and a peptide located at the thrombin cleavage site on the extracellular portion of the thrombin receptor (mAb-TRC). *In vitro*, both antibodies inhibited thrombin-induced human platelet aggregation (results not shown). As shown in Figure 4, the mitogenic effect of TRAP was totally abolished by a specific anti-TRAP antibody, whereas TRAP-induced HUVEC growth was

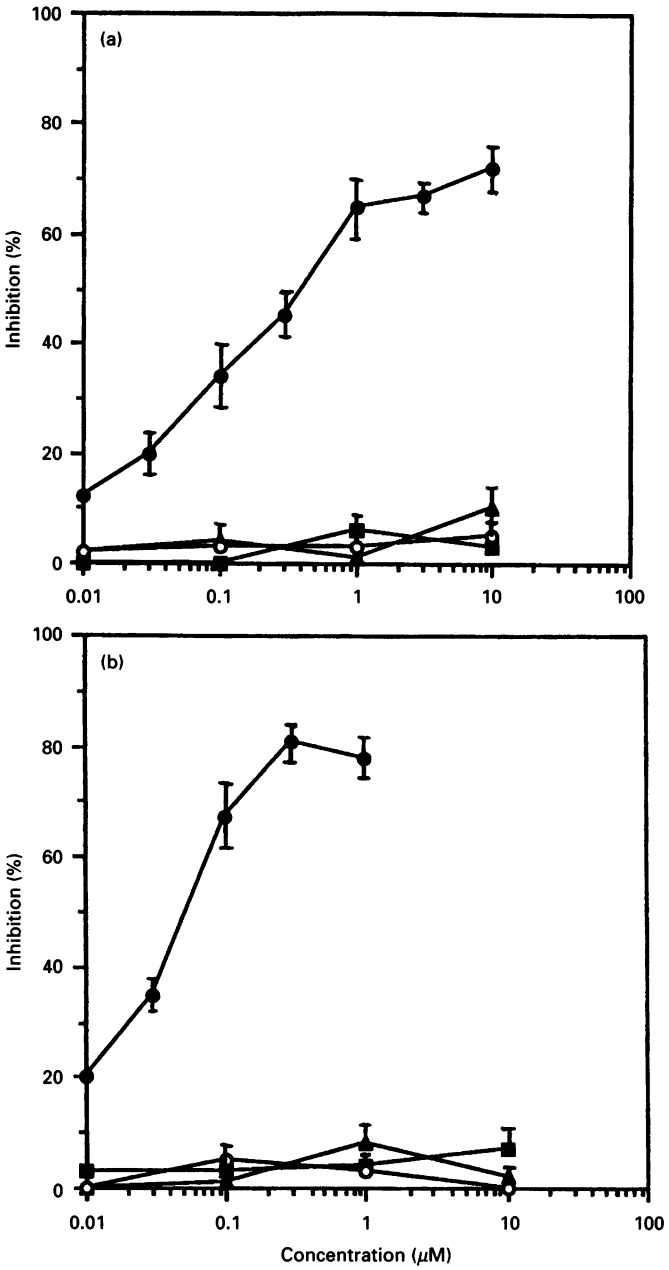

Figure 3 Effect of thrombin inhibitors on HUVEC growth

HUVECs (1×10^4 cells/well) were allowed to grow in the presence of culture medium containing 0.5% FCS supplemented with α-thrombin (1 μM) (●), TRAP (1 μM) (■), ECGS/heparin (30 μg/ml + 100 μg/ml) (▲) or F-14 (10 μM) (○). Increasing concentrations of NAPAP (**a**) or hirudin (**b**) were added simultaneously. After 5 days in culture, triplicate wells were trypsinized and cells were counted. Data are expressed as mean percentage inhibition ± S.D. of proliferation compared with replicate cultures grown without the inhibitor ($n = 9$).

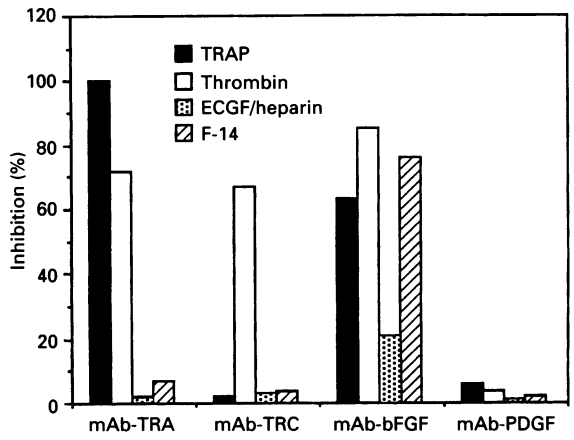

Figure 4 Effect of various antibodies on thrombin-, TRAP- and F-14-induced HUVEC growth

HUVECs (1×10^4 cells/well) were allowed to grow in the presence of culture medium containing 0.5% FCS supplemented with TRAP (1 μM) (closed bars), α-thrombin (0.1 μM) (open bars), F-14 (10 μM) (hatched bars) or ECGF/heparin (stippled bars). Monoclonal mouse anti-(bovine bFGF) antibody (mAb-bFGF), monoclonal mouse anti-(human PDGF-BB) antibody (mAb-PDGF), neutralizing antisera directed against TRAP and TRC (KATNATLDPR), a decapeptide adjacent to the cleavage site on the extracellular portion of the thrombin receptor, raised in the goat (dilution factor: 1/100) were added simultaneously. After 5 days in culture, triplicate wells were trypsinized and cells were counted. Data are expressed as mean percentage inhibition of proliferation compared with replicate cultures grown without the antibodies ($n = 9$).

not affected by mAb-TRC. These two antibodies, mAb-TRAP and mAb-TRC, inhibited only partially the native α-thrombin-induced proliferation of HUVECs (Figure 4) further showing that the mitogenic effect of thrombin with regard to HUVECs is only partially mediated by the proteolytic pathway. The mitogenic effect of ECGF/heparin was not altered by mAb-TRAP or mAb-TRC, showing that both antibodies were specifically affecting the mitogenic signal of thrombin via its cell-surface receptor. Under the same experimental conditions, F-14 retained full mitogenic effect when co-incubated either in the presence of mAb-TRAP or mAb-TRC, therefore showing that it interacts with different receptor sites on HUVEC cell membranes.

In a recent paper, Weiss and Maduri [21] showed that the mitogenic effect of thrombin for vascular smooth muscle cells was due to the release of bFGF by the cells. In order to determine if such an effect was encountered for HUVECs as well, we evaluated the activity of a neutralizing monoclonal anti-bFGF antibody (mAb-bFGF). mAb-bFGF inhibited the HUVEC growth induced by either thrombin, TRAP or F-14 (63%, 85% and 76% inhibition respectively) (Figure 4). This inhibitory effect did not occur with regard to ECGF/heparin-induced HUVEC growth and was not a general phenomenon involving all tyrosine kinase-coupled receptor agonists as we observed no significant inhibitory effect of a monoclonal antibody directed against PDGF-BB (mAb-PDGF) on thrombin-, TRAP- or F-14-induced HUVEC growth (Figure 4).

Therefore, our results demonstrate for the first time that thrombin-induced HUVEC growth occurs via two different pathways, both of which act with bFGF as an essential intermediary; however, the precise mechanism by which thrombin, and more specifically the loop-B region, modulates endothelial cell proliferation remains to be fully elucidated.

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