Characterization of Thrombin Receptor Expression During Vascular Lesion Formation

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Abstract Blood vessels respond to injury by initiating cell proliferation and migration that result in vascular lesion formation. To determine the roles of thrombin and the thrombin receptor in this process, we characterized thrombin receptor expression in normal and injured arteries, thrombin receptor-mediated smooth muscle cell mitogenesis, and the regulation of thrombin receptor mRNA expression in vitro. Thrombin receptor mRNA was not detected in normal rat or baboon arteries by in situ hybridization. Immunohistochemistry using an anti-thrombin receptor antibody (TR-R9), directed against the thrombin cleavage site of the rat aortic smooth muscle cell thrombin receptor, revealed low-level staining for thrombin receptor protein in endothelial cells and smooth muscle cells of normal arteries. In contrast, balloon catheter injury increased thrombin mRNA expression in medial smooth muscle cells within 6 hours. This increased thrombin receptor expression continued within the media and in neointimal cells throughout vascular lesion formation, predominantly in areas of active cell proliferation. In vitro, α-thrombin stimulates rat aortic smooth muscle cell proliferation in a concentration-dependent manner.

Restenosis is an accelerated form of vascular lesion formation that develops after percutaneous transluminal coronary angioplasty. The sequence of events giving rise to vascular lesion formation after balloon catheter denudation—type angioplasty has been studied extensively in experimental animals. Typically the “first wave” of smooth muscle cell proliferation occurs in the media within 36 to 48 hours, followed by migration of smooth muscle cells across the internal elastic lamina.1,2 Subsequently, cell proliferation occurs in the neointimal smooth muscle cells and may continue for up to 3 months. In vitro and in vivo studies implicate basic fibroblast growth factor, platelet-derived growth factor (PDGF), and thrombin in this process.3-5

Since thrombin is present during arterial thrombosis and after balloon catheter injury,6 it may be an important mediator of vascular lesion formation. Two recent observations support this hypothesis: (1) Thrombin receptor activation stimulates vascular smooth muscle cell proliferation in vitro.7 (2) There is increased expression of the thrombin receptor in advanced atherosclerotic lesions.8 To further understand the role of thrombin and thrombin receptor activation in vascular lesion formation, we characterized thrombin receptor expression in vascular smooth muscle cells throughout the period of vascular lesion formation in rat and baboon arterial injury models. In addition, we investigated thrombin receptor activation during thrombin-mediated smooth muscle cell mitogenesis in vitro and the effects of growth factors thought to be important in vascular lesion formation, basic fibroblast growth factor and PDGF, on thrombin receptor expression.

Materials and Methods

Thrombin Receptor Antibody

A polyclonal anti-thrombin receptor antibody (TR-R9) specific for the rat aortic smooth muscle cell thrombin receptor was raised by immunizing rabbits with the synthetic oligopeptide SFFLRNPSEC coupled to keyhole limpet hemocyanin by using bromoacetate. This peptide sequence was selected because it represents the “new amino terminus” resulting from the cleavage by thrombin of the extracellular amino terminus of the rat aortic smooth muscle cell thrombin receptor.9,10 New Zealand White rabbits were immunized, boosted, and bled as described previously.11 TR-R9 antibodies were purified by affinity chromatography using SFFLRNPSEC-Sepharose.
To confirm the specificity of TR-R9, Western blots were performed by using selected sequences from pRTHR17 (containing the entire cDNA of the rat vascular smooth muscle cell thrombin receptor) amplified by polymerase chain reaction. The amplified products were ligated into pQE vectors by using the QIAexpress system (QIAGEN Inc), expressed in Escherichia coli, and purified from cell supernatant by making use of the 6xHis affinity tag coding sequence. A single-step purification using Ni-NTA resin yielded >90% pure protein. Western blot analysis was then performed by using TR-R9, Ang215 (an antibody specific for the amino terminus of the rat angiotensin II AT1 receptor14), and TR309 (an antibody specific for the third intracellular domain of the rat smooth muscle cell thrombin receptor); ECL Western blotting protocol (Amer sham) was used.

**Thrombin Purification and Characterization**

Human α-thrombin was purified from fresh frozen human plasma by using the method of Lundblad et al.12 Purified α-thrombin was characterized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and activity was determined and confirmed by chromogenic substrate assay (S-2238) and fibrinogen clotting assays.12

**In Vitro [3H]Thymidine Incorporation Assay**

Rat aortic smooth muscle cells were isolated by using a method adapted from Travo et al.13 In brief, aortas were dissected from normal 250- to 300-g Sprague-Dawley male rats. After mechanical removal of adventitia and endothelium, rat aortic smooth muscle cells were obtained by digestion of aortas with collagenase and elastase. The identity of cultured cells as smooth muscle cells was confirmed by anti-α-smooth muscle actin antibody staining (anti-α-sm-1, Sigma Chemical Co). The protocol used to study the effect of thrombin on rat aortic smooth muscle cell proliferation in vitro was adapted from that of Gasic et al.14 Early passage (before passage 6) rat aortic smooth muscle cells in Dulbecco’s modified Eagle’s medium (DME) with 10% fetal bovine serum (Whitaker) were washed twice with DME without serum and then incubated with DME containing 0.1% bovine serum albumin (BSA, Sigma) for 24 hours. The DME/BSA buffer was then replaced with DME containing either human α-thrombin (10 to 140 nmol/L), the peptide agonist SFFLRN (1 nmol/L to 100 μmol/L), 0.1% BSA, or 10% fetal bovine serum. In some experiments, cells were incubated with TR-R9 (100 μg/mL) for 30 minutes before the addition of α-thrombin, SFFLRN, fetal bovine serum, or BSA to determine if there was any effect on cell proliferation. In these experiments, the concentration of TR-R9 used (100 μg/mL) was the optimal concentration selected from a dose-response experiment using this antibody to inhibit thrombin-induced calcium mobilization in fura 2-loaded rat aortic smooth muscle cells (not shown). After 48 hours, the cultures were washed twice with DME and pulsed for 2 hours with 1 μCi/ml [3H]thymidine (1740 GBq/mmol, New England Nuclear) in DME containing 0.1% BSA. Cells were washed with cold phosphate-buffered saline, treated with 5% trichloroacetic acid for 5 minutes, dissolved in 0.25 mol/L NaOH, mixed with scintillation fluid, and counted. Assays were performed in triplicate.

**Northern Blots**

To determine the effect of growth factors on thrombin receptor expression, Northern blot analysis was performed by using mRNA isolated from rat aortic smooth muscle cells treated with serum, basic fibroblast growth factor, and PDGF-AA. Early-passage rat aortic smooth muscle cells were growth-arrested by replacing serum-containing culture medium with DME containing 1% human platelet-poor plasma for 48 hours. Either basic fibroblast growth factor at a final concentration of 20 ng/mL or PDGF-AA at a final concentration of 100 ng/mL (both from Biomedical Technologies) was then added to the culture medium. For positive and negative controls, the culture medium was replaced with DME containing either 10% or 0.1% fetal bovine serum. Cells were harvested at 1, 2.5, 4, 8, and 24 hours, and poly(A) mRNA was prepared by CsCl ultracentrifugation and oligo dT-cellulose chromatography. After transfer to a nitrocellulose filter, hybridization to a [32P]dCTP-labeled PsI I fragment of the rat aortic smooth muscle cell thrombin receptor (pRTHR17)9 was performed under high stringency (washing with 0.1× standard saline citrate [SSC] and 0.1% SDS at 65°C for 1 hour). The amount of RNA loaded onto gels was quantified by spectrophotometry before loading and by methylene blue staining after transfer for each experiment. After hybridization with the thrombin receptor probe, filters were hybridized with a [3P]-labeled 1.5-kb PsI fragment of the constitutively expressed gene15 glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Northern blot analysis of early-passage rat aortic smooth muscle cells shows a single 3.45-kb thrombin receptor mRNA band. Thrombin receptor and GAPDH mRNA levels were quantified by densitometry.

**In Situ Hybridization**

Thrombin receptor in situ hybridization was performed by using riboprobes transcribed16 with [35S]UTP (specific activity, 1200 Ci/mmol; Amersham) from either a 1.2-kb PsI I fragment of pRTHR17 that contained most of the coding sequence of the rat thrombin receptor or a 596-bp fragment of the human thrombin receptor cDNA generated from nucleotides 286 to 882 of the human sequence.9 PDGF-A chain in situ hybridization was performed on rat and primate vessels by using a 900-bp EcoRI fragment of mouse PDGF-A cloned into pGEM7 (gift from M. Mercola and C. Stiles) and a 1280-bp EcoRI-HindIII fragment of human PDGF-A10 (gift from C. Betsholtz) subcloned into sp64, respectively.

In situ hybridization was performed as previously described16–21 by using 35S-labeled sense and anti-sense riboprobes. Briefly, cryosections were pretreated with proteinase K (Sigma) and prehybridized in 100 μL hybridization buffer (50% formamide, 0.3 mol/L NaCl, 20 mmol/L Tris [pH 8.0], 5 mmol/L EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% BSA, 10% dextran sulfate, and 10 mmol/L dithiothreitol) at 42°C. The hybridizations were performed by using 600 000 cpm of 35S-labeled riboprobe at 55°C. After hybridization, the sections were washed with 2× SSC (1× SSC contains 150 mmol/L NaCl and 15 mmol/L sodium citrate [pH 7.0]) with 10 mmol/L β-mercaptoethanol and 1 mmol/L EDTA, followed by 1× SSC containing 0.1% sodium dodecyl sulfate (NaDodSO4) and again washed in the same buffer, followed by a high-stringency wash in 0.1× SSC with 10 mmol/L β-mercaptoethanol and 1 mmol/L EDTA, at 52°C. The slides were then washed in 0.5× SSC and dehydrated in graded alcohols containing 0.3 mol/L NaHAc. The sections were dried, coated with NTB2 nuclear track emulsion (Kodak), and exposed in the dark at 4°C for 4 to 12 weeks. After development, the sections were counterstained with hematoxylin and eosin to aid in cell identification.

**Immunohistochemistry**

Immunohistochemistry was performed with the rabbit polyclonal antibody TR-R9 at a concentration of 5 μg/mL by using the Vectastain ABC alkaline phosphatase system (Vector Labs). The final reaction product was stained with alkaline phosphatase substrate kit 1 to give a final stain that appeared red, and tissues were counterstained with methyl green or hematoxylin. Parallel sections treated with secondary antibody only, with nonimmune rabbit IgG (Vector) at the same concentration as TR-R9, or with TR-R9 and a 100-fold excess of the peptide used to generate this antibody (SFFLRNSPEC) were used as a control and showed no specific staining. Proliferating cells were labeled by immunohistochemistry using...
ing an antibody directed against proliferating cell nuclear antigen (Oncogene Science) and ABC alkaline phosphatase staining. Smooth muscle cells were identified in tissue sections by using antibody HHF35 (Enzo Diagnostics).

Surgery/Tissue Preparation

The distribution of cells containing thrombin receptor mRNA and protein were examined after denudation-type balloon angioplasty of rat carotid arteries. Sprague-Dawley rats (250 to 300 g, n=3 for each time point) were anesthetized, and a 2F Fogarty embolectomy catheter was introduced into the left carotid artery via the left femoral artery. The balloon was inflated with 50 μL of water and withdrawn slowly three times. The completeness of denudation was verified in a parallel group of animals by injecting Evans blue dye immediately after withdrawing the catheter and histological examination. Tissues were examined for thrombin receptor mRNA and protein at 6 hours, 1 day, 3 days, 1 week, and 2 weeks after balloon catheter angioplasty of rat carotid arteries. The contralateral uninjured carotid was collected from each animal and served as a control at each time point. Vascular lesion formation was induced in normal baboon carotid arteries by surgical endarterectomy. Endarterectomy specimens were harvested 6 hours or 30 days after surgery and examined for thrombin receptor and PDGF-A chain mRNA expression by in situ hybridization. All animal experimentation was reviewed and approved by the Emory University and Yerkes Primate Research Center institutional animal care and use committees.

Tissues were obtained fresh and fixed in 4% paraformaldehyde buffered with 0.1 mol/L NaPO₄, (pH 7.4) for in situ hybridization, or methyl Carnoy’s fixative for proliferating cell nuclear antigen immunohistochemistry, for 3 to 4 hours at 4°C, cryoprotected in 15% sucrose–phosphate-buffered saline overnight, embedded in optimal cutting temperature compound (O.C.T., Miles Laboratories), frozen in liquid nitrogen, and stored at ~70°C. Cryosections (7 to 10 μm) were thaw-mounted onto slides coated with Vectabond (Vector Laboratories) or Superfrost/Plus slides (Fisher Scientific), refrozen, and stored at ~70°C with desiccant until used.

Results

Specificity of the Thrombin Receptor Antibody TR-R9

We confirmed the specificity of TR-R9 for the rat aortic smooth muscle cell thrombin receptor by several independent approaches. Western blot analysis was performed with two fusion proteins to confirm the specificity of TR-R9. The first fusion protein consisted of amino acids 1 to 100 of the rat smooth muscle cell thrombin receptor, and the second consisted of amino acids 42 to 100 (beginning with Ser, the new amino terminus after thrombin cleavage). TR-R9 binds to both fusion proteins (Fig 1). However, as anticipated, neither Ang215 nor TR309 bound to either fusion protein, since Ang215 is specific for an entirely different protein and TR309 is specific for a sequence of the rat aortic smooth muscle cell thrombin receptor not present in either fusion protein.

In the second approach, TR-R9 was demonstrated to neutralize thrombin action on cultured rat smooth muscle cells. TR-R9, but not control antibodies or preimmune sera, inhibited thrombin-induced intracellular calcium mobilization in thrombin-treated rat aortic smooth muscle cells (K. Wick, K.K. Grindling, and M.S. Runge, unpublished data).

The specificity of TR-R9 was further supported by a number of experiments that demonstrated appropriate immunohistochemistry responses. TR-R9 showed specific membrane and intracellular localization of the thrombin receptor in cultured paraformaldehyde-fixed rat aortic smooth muscle cells by using TR-R9 and fluorescence microscopy (not shown). Tissue localization of TR-R9 was confirmed by staining serial sections with nonimmune IgG or an irrelevant antibody (see Fig 3). In addition TR-R9 staining in the rat and primate tissues could be eliminated by competition with free peptide. Before addition to the tissue sections, TR-R9 was incubated for 1 hour at room temperature with a 100-fold molar excess of the synthetic oligopeptide SFFLRNPSEC, which was used to raise the antibody in rabbits. Preincubation of TR-R9 with SFFLRNPSEC competed out TR-R9 staining in both the rat and baboon vessels (not shown).

Thrombin Receptor Expression in Medial Smooth Muscle and Neointimal Cells During Vascular Lesion Formation in Rat Carotid Artery

Studies of thrombin receptor mRNA in normal rat carotid arteries by in situ hybridization demonstrated no detectable thrombin receptor mRNA in the media (Fig 2A). Thrombin receptor mRNA-containing cells were found in the adventitia surrounding normal arteries. Immunohistochemistry for thrombin receptor protein in normal vessels using the polyclonal antibody TR-R9 showed staining of medial smooth muscle cells and of the endothelium (Fig 3A). These findings, especially the localization of protein in endothelial and medial cells without detectable mRNA, suggest a very low rate of turnover of the thrombin receptor in normal quiescent arterial endothelial and smooth muscle cells.

The expression of thrombin receptor mRNA in the rat carotid artery was increased after balloon catheter injury. As early as 6 hours after injury thrombin receptor mRNA was detected throughout the media and in the adventitia (Fig 2B). In situ hybridization also showed a corresponding increase in PDGF-A chain mRNA in the medial cells (Fig 2C), consistent with the
finding of increased expression of PDGF-A chain mRNA by Northern blot analysis at this time. Two weeks after balloon catheter angioplasty, thrombin receptor mRNA–containing cells were found diffusely distributed throughout the neointima and the media (Fig 2D), whereas PDGF-A synthesis was confined to the neointimal cells at this time (data not shown).

No specific changes in thrombin receptor protein staining after balloon injury of the rat carotid artery could be detected by immunohistochemistry. Thrombin receptor protein staining was consistently found in the media of the carotid artery during the first 7 days after balloon angioplasty. Two weeks after angioplasty, thrombin receptor protein was diffusely distributed throughout the neointima and was also present in the underlying medial cells (Fig 3).

**Thrombin Receptor Expression After Endarterectomy of Baboon Carotid Arteries**

Thrombin receptor mRNA was not detected by in situ hybridization in normal baboon carotid arteries (Fig 4A). However, immunohistochemistry on these vessels using TR-R9 indicated that there was low-level protein staining of the medial and endothelial layers similar to that seen in the rat (not shown). Thrombin receptor mRNA–containing cells were found in vascular lesions, which formed 30 days after surgical endarterectomy of the baboon carotid artery, localized to smooth muscle cells underlying the endothelial surface (Fig 4). In these vessels, PDGF-A and thrombin receptor mRNAs were colocalized in regions of active smooth muscle cell proliferation, as indicated by adjacent segments of these vessels stained for PCNA (Fig 5). PDGF mRNA was
also found in the endothelial cells over these lesion sites but not in regions of normal nonendarterectomized carotid proximal or distal to the injury site (not shown).

Effect of Anti-Thrombin Receptor Antibody TR-R9 on [\(^3\)H]Thymidine Uptake Induced by \(\alpha\)-Thrombin

In vitro studies indicate that thrombin or the thrombin receptor agonist peptide stimulates mitogenesis in arterial smooth muscle cells.\(^{2,25}\) In the present series of experiments, we set out to prove that this was due to stimulation of the thrombin receptor by demonstrating an inhibition of mitogenesis by the receptor antibody TR-R9. We used [\(^3\)H]thymidine incorporation in smooth muscle cells in vitro as an index of thrombin-induced mitogenesis. In these experiments, the maximal stimulation of [\(^3\)H]thymidine incorporation in early-passage rat aortic smooth muscle cells occurs at \(\approx\)10 nmol/L \(\alpha\)-thrombin. The thrombin receptor agonist peptide SFFLRN is at least as potent a mitogen as \(\alpha\)-thrombin in the absence of additional growth factors, although higher concentrations of SFFLRN are typically required for maximal mitogenic stimulation (50 to 100 \(\mu\)m). Preincubation of rat aortic smooth muscle cells with TR-R9 before the addition of \(\alpha\)-thrombin results in a marked decrease in thrombin-induced [\(^3\)H]thymidine incorporation (Fig 6). However, TR-R9 has no effect on SFFLRN-induced smooth muscle cell [\(^3\)H]thymidine incorporation. We hypothesize that receptor inhibition by TR-R9 results from a blockade of receptor cleavage and not from inhibiting interaction of the tethered ligand with the “ligand binding pocket” of the receptor. Thus, the lack of an effect of TR-R9 on peptide-induced [\(^3\)H]thymidine incorporation was not unexpected. These data indicate that in rat aortic smooth muscle cells, the mitogenic effect of \(\alpha\)-thrombin is mediated via activation of the smooth muscle cell thrombin receptor.

Regulation of Thrombin Receptor mRNA In Vitro by Basic Fibroblast Growth Factor and PDGF-AA

PDGF and basic fibroblast growth factor are important growth factors that have been implicated in vascular lesion formation after mechanical injury.\(^{2,4,26}\) To investigate the possible interaction of these growth factors with thrombin, we examined their effect on thrombin receptor expression in vitro. Quiesced rat aortic smooth muscle cells were treated with either basic fibroblast growth factor (10 nmol/L) or PDGF-AA (20 nmol/L). Cells were harvested, and mRNA was isolated over a 24-hour period after growth factor addition. Thrombin receptor mRNA levels were determined by Northern analysis.

Treatment of rat aortic smooth muscle cells with either basic fibroblast growth factor or PDGF-AA caused an absolute increase in thrombin receptor mRNA, based on measurement of total RNA. In addition, a relative increase in thrombin receptor mRNA was found compared with that of GAPDH in multiple experiments conducted with different preparations of

Fig 3. Photomicrographs showing immunohistochemical localization of thrombin receptor protein in the rat carotid artery after vascular injury. Thrombin receptor immunohistochemistry was performed using the anti-thrombin receptor antibody TR-R9: A shows normal carotid artery; B, carotid artery 1 week after balloon angioplasty; C, carotid artery 2 weeks after balloon angioplasty; and D, carotid artery 2 weeks after balloon angioplasty, stained with the same concentration of a nonimmune IgG rather than with TR-R9. The arrow indicates the border of the internal elastic lamina in panels C and D (original magnification \(\times 100\)).
FIG 4. Photomicrographs showing localization of thrombin receptor mRNA by in situ hybridization in the normal baboon carotid artery and 30 days after surgical endarterectomy. Thrombin receptor mRNA was not detected in normal carotid arterial media but was localized to adventitial fibroblasts (arrows, A). Surgical endarterectomy and carotid artery harvesting 4 weeks after injury were performed as previously described.23 Thirty days after carotid endarterectomy, thrombin receptor mRNA-containing smooth muscle cells were found along the lumenal surface just beneath the endothelium (B). The in situ hybridization experiments were controlled by hybridizing serial sections with 35S-labeled thrombin receptor riboprobes transcribed in the sense orientation. The sense hybridizations were negative (C). Smooth muscle cells were identified in these tissues by immunohistochemistry using the antibody HHF-35 (D). Arrowheads indicate border of residual media and the beginning of the neointima (original magnification x50).

rat aortic smooth muscle cells. The time course for induction of thrombin receptor mRNA expression was different for these two growth factors: basic fibroblast growth factor increased thrombin receptor mRNA 1.8-fold by 1 hour with a sustained 1.5-fold increase at 8 and 24 hours (Fig 7), whereas PDGF-AA caused an initial decrease in thrombin receptor mRNA levels, followed by a more marked increase (2.4-fold) that was sustained at 8 and 24 hours. Although these effects are relatively small, they are reproducible. Absolute determinations of thrombin receptor numbers on these cells are not currently feasible. Unfortunately, neither functional assays nor binding assays are sufficiently sensitive to determine whether these differences in thrombin receptor mRNA are also present at the protein level. Interestingly, thrombin itself has little or no effect on thrombin receptor mRNA levels in these preparations (not shown). A single administration of 140 nmol/L α-thrombin does not significantly alter thrombin receptor mRNA levels at 8 and 24 hours.

Discussion

The signals inducing the phenotypic modulation of medial smooth muscle cells after mechanical vascular injury have not been fully characterized. Many potential stimuli, including cytokines, growth factors, physical factors, and other complex mediators, have been examined. Since thrombin is present acutely at the time of mechanical injury and high levels persist throughout the period of vascular lesion formation, we hypothesized that thrombin might be an important mediator of this process. The overall goal of these studies therefore was
to determine whether thrombin may be involved in the process of vascular lesion formation by analyzing the distribution of cells containing a thrombin receptor that has been previously linked to thrombin-induced mitogenesis in vascular smooth muscle cells.\(^7\)

In situ hybridization and immunohistochemical data from the present study demonstrate that upregulation of smooth muscle cell thrombin receptor expression in vivo is a very early event in vascular lesion formation and that increased thrombin receptor expression continues throughout neointimal development. Thrombin receptor and PDGF-A chain mRNAs are found colocalized at all times after vascular injury and are found at sites consistent with the localization of proliferating smooth muscle cells during the later phases of vascular lesion formation. The colocalization of thrombin receptor and PDGF mRNAs at sites of smooth muscle cell proliferation suggests that thrombin may continue to exert a mitogenic effect at these times. Two growth factors with established roles in vascular lesion formation, basic fibroblast growth factor and PDGF, upregulate thrombin receptor expression as determined by Northern blot analysis and may be involved in the initiation and maintenance of thrombin receptor synthesis throughout lesion development.

Thrombin is known to be a mitogen for a number of cell types in vitro, including fibroblasts and vascular smooth muscle cells.\(^7,25,27-28\) Although thrombin can potentially interact with a number of cell surface proteins, it has been hypothesized that the mitogenic effect of thrombin is due to activation of the cellular thrombin receptor. The strongest indirect support for this hypoth-

Fig 5. Photomicrographs showing localization of thrombin receptor and platelet-derived growth factor (PDGF)-A mRNA in proliferating smooth muscle cells 30 days after carotid endarterectomy of the baboon. Serial sections were analyzed for thrombin receptor (A) or PDGF-A (B) mRNAs by in situ hybridization in paraformaldehyde-fixed vessel segments. Proliferating cells were identified by proliferating cell nuclear antigen immunohistochemistry on Carnoy's fixed vessel segments adjacent to those used for in situ hybridization (C). Thrombin receptor mRNA-containing cells were detected in the neointima just beneath the luminal surface (A). These were identified as smooth muscle cells on the basis of serial section immunohistochemistry using smooth muscle-specific antibody HGF35 (see Fig 4D). PDGF-A mRNA was localized to endothelial cells on the surface of the lesion and to smooth muscle cells just beneath the endothelium (B). PDGF-A and thrombin receptor mRNA localization corresponded to sites of cellular proliferation identified by proliferating cell nuclear antigen staining (arrows, C) (original magnification ×125).

Fig 6. Bar graph showing inhibition of α-thrombin-mediated vascular smooth muscle cell mitogenesis by anti-thrombin receptor antibody TR-R9. Early-passage (before passage 6) rat aortic smooth muscle cells were grown to 50% to 70% confluence in DME with 10% fetal bovine serum (FBS, Whittaker). After 24 hours of quiescence in DME containing 0.1% bovine serum albumin (BSA), the cells were treated with either human α-thrombin (10 nmol/L), the peptide agonist SFFLRN (25 μmol/L), 0.1% BSA, or 10% FBS. In parallel experiments, before the addition of α-thrombin or SFFLRN, cells were incubated with TR-R9 (100 μg/mL for 30 minutes) to determine if there was any effect on cell proliferation. No effect was seen. After 48 hours, the cultures were washed twice with DME and pulsed for 2 hours with [\(^{3}H\)]thymidine in DME containing 0.1% BSA. Each bar represents the mean±SD of three separate experiments.
Fig 7. Graph showing basic fibroblast growth factor and platelet-derived growth factor (PDGF-AA) stimulation of thrombin receptor (TR) mRNA expression. Shown here are mean±SEM values resulting from five independent Northern blot experiments with basic fibroblast growth factor stimulation (C) and three independent experiments with PDGF stimulation (A), performed as described in “Materials and Methods.” TR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were quantified by densitometry, and changes in TR mRNA levels relative to GAPDH mRNA levels are shown here.

Discussion

The observation that the thrombin receptor agonist peptide SFFLRN stimulates mitogenesis in some, but not all, systems. Recent reports indicate that inhibition of Gαs and Gα13 inhibits thrombin- and/or SFFLRN-induced mitogenesis. The inhibition of thrombin receptor activation by the blocking antibody TR-R9 reported here is a direct demonstration that activation of the thrombin receptor is responsible for the mitogenic effect of thrombin on vascular smooth muscle cells. This has been further confirmed by more recent work indicating that inhibition of thrombin receptor synthesis in vitro by anti-sense oligonucleotides also blocks thrombin-induced mitogenesis (M. Runge, unpublished data).

The unique aspect of these data is the use of well-characterized animal models to study the role of thrombin receptor expression during vascular lesion formation. In a study of advanced athersclerotic plaque, Nelken et al. demonstrated thrombin receptor expression in tissue macrophages by in situ hybridization and in vascular smooth muscle cells by Northern blot analysis. They postulated that the relatively few vascular smooth muscle cells found to express thrombin receptor mRNA in complex atheroma might represent a subset of migrating cells. In contrast, our data show that as early as 6 hours after injury, there is an increase in thrombin receptor mRNA in medial smooth muscle cells, indicating that thrombin receptor expression is upregulated before cell division or migration. Furthermore, the data presented here indicate that the effects of thrombin on vascular cell proliferation are receptor-mediated. McNamara et al. previously demonstrated that both α-thrombin and SFFLRN were mitogenic, consistent with the hypothesis that thrombin receptor activation on smooth muscle cells initiates proliferation. Our data confirm the role of thrombin receptor activation in mitogenesis and extend this with our in vivo observations indicating that there is increased expression of thrombin receptor mRNA at sites within experimental vascular lesions that are typically associated with smooth muscle cell proliferation.

It is unlikely that a single mitogen or cytokine is responsible for the initiation and maintenance of vascular lesion formation. The development of selective strategies for inhibiting lesion development therefore necessitates an understanding of the mechanisms that regulate vascular cell proliferation. These data suggest that an important role of both basic fibroblast growth factor and PDGF in vascular injury is the stimulation of thrombin receptor expression. This is consistent with observations of a cooperative effect between basic fibroblast growth factor and SFFLRN action in CCL-39 cell mitogenesis and is of particular importance in light of a recent report on the kinetics of thrombin receptor activation. Ishii et al. proposed that each cleaved thrombin receptor generates a quantum of second messenger and that the magnitude of the response of the cell to thrombin depends on the absolute amount of second messenger present at a given time and hence on the ratio between second-messenger generation and clearance. The rapidity of second-messenger generation is related to the local concentration of thrombin but is dependent on receptor number. Thus, increased thrombin receptor gene expression in injured vascular wall cells may allow more marked mitogenic responses to α-thrombin.

Reciprocal interactions between thrombin, fibroblast growth factor, and PDGF may be important during vascular lesion formation. This hypothesis is supported by in vitro studies indicating that thrombin promotes the synthesis and release of PDGF in mesangial, smooth muscle, and endothelial cells. Furthermore, inhibition of thrombin activity using the irreversible anti-thrombin peptide D-Phe-Pro-Arg chloromethyl ketone prevents thrombin-induced expression of PDGF-A mRNA by smooth muscle cells in vitro as well as the early expression of PDGF-A mRNA, which normally increases by 6 hours after balloon catheter angioplasty of baboon brachial arteries in vivo. Thrombin also promotes the synthesis and action of basic fibroblast growth factor in smooth muscle cells, and thrombin-induced mitogenesis in smooth muscle cells in vitro is partially blocked by antibodies directed against either PDGF-AA or basic fibroblast growth factor. Therefore, the induction of basic fibroblast growth factor and PDGF synthesis in smooth muscle cells appears to be essential for thrombin to express its full mitogenic potential and thrombin action may be important in maintaining PDGF-A and basic fibroblast growth factor synthesis in vivo after vascular injury.

Basic fibroblast growth factor, PDGF, and thrombin are all likely to be involved in the proliferative growth response to balloon injury. Antibodies against basic fibroblast growth factor prevent early medial smooth muscle replication that occurs 24 to 48 hours after balloon injury. Antibodies against platelets or PDGF induce a statistically significant reduction in neointimal development after balloon injury by apparently blocking migration of smooth muscle cells from the media to the neointima. Hirudin, a specific thrombin antagonist, is effective in preventing restenosis in a cholesterol-fed rabbit model subjected to balloon angioplasty when administered at the time of injury. However, the
model used was a complex multiple-injury study of atherosclerotic vessels that relied on morphological end points, so it is not clear whether hirudin acted by reducing the amount of thrombosis at the site of injury or by inhibiting smooth muscle cell proliferation directly. Recent studies of balloon angioplasty in primates indicate that continuous administration of high doses of hirudin inhibits the first wave of smooth muscle cell proliferation and PDGF-A expression that normally occurs 3 days after injury (J.N. Wilcox, L.A. Harker, and S.R. Hanson, unpublished data). The question of thrombin action on smooth muscle cell proliferation during the later phases of lesion development is currently under investigation.

In summary, we hypothesize that after balloon catheter angioplasty, basic fibroblast growth factor begins the proliferative process and stimulates thrombin receptor synthesis. Fibroblast growth factor action may be amplified by autocrine production of basic fibroblast growth factor induced by the action of thrombin on these cells. Inhibition of basic fibroblast growth factor therefore inhibits early medial smooth muscle cell proliferation and may prevent acute thrombin receptor and PDGF gene expression. Subsequently, thrombin is important in maintaining the proliferative process and the autocrine production of PDGF-A by proliferating smooth muscle cells. PDGF expressed by proliferating smooth muscle cells, in turn, maintains thrombin receptor gene expression throughout neointimal development. Basic fibroblast growth factor has little role in the later phases of lesion development, which may instead be driven in large part by thrombin. Although this interpretation is supported by studies indicating that blockage of basic fibroblast growth factor action as late as 2 weeks after balloon catheter angioplasty of the rat carotid has no effect on continuing cell proliferation in the neointima, additional studies are needed to determine what effect the inhibition of thrombin at later times might have on cell proliferation and lesion development. In 1852, von Rokitansky (Schwartz et al) suggested that human atherosclerotic plaques may represent the abnormal deposition of fibrin or blood products on the surface of blood vessels, giving rise to the thrombogenic or encrustation hypothesis for atherosclerosis. The data presented here suggest an important role for thrombin in the initiation and maintenance of the proliferative process that occurs after vascular injury.

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