Thrombin Receptor Expression in Normal and Atherosclerotic Human Arteries

Nicolas A. Nelken,*‡ Scott J. Soifer,*§ Julie O'Keefe,* Thien-Khai H. Vu,* Israel F. Charo,\|¶ and Shaun R. Coughlin*\|
**Cardiovascular Research Institute and Departments of ‡Surgery, §Pediatrics, and \|Medicine, and ¶Gladstone Institute of Cardiovascular Disease, University of California, San Francisco, California 94143*

Abstract

Thrombin is a multifunctional serine protease generated at sites of vascular injury. A host of thrombin actions on vascular endothelial cells, smooth muscle cells, and macrophages has been defined in cell culture systems, but the in vivo significance of these activities is unknown. We have defined the expression of the recently identified receptor for thrombin in human arteries by both in situ hybridization and immunohistochemistry. In normal-appearing arteries, thrombin receptor was expressed almost exclusively in the endothelial layer. By contrast, in human atheroma, the receptor was widely expressed, both in regions rich in macrophages and in regions rich in vascular smooth muscle cells and mesenchymal-appearing intimal cells of unknown origin. Thrombin receptor was expressed by human vascular endothelial cells and smooth muscle cells in culture and by macrophages obtained by bronchioalveolar lavage, thus demonstrating that all three cell types are indeed capable of expressing the thrombin receptor. These results establish thrombin receptor activation as a candidate for contributing to sclerotic and inflammatory processes in the human vasculature, such as those that occur in atherosclerosis and restenosis. (*J. Clin. Invest.* 1992.90:1614–1621.) Key words: thrombin receptor • *Xenopus* oocytes • expression cloning • signaling • platelet

Introduction

Thrombin, a multifunctional serine protease generated at sites of vascular injury, exhibits a host of cell activating functions (reviewed in references 1–3). It is the most potent activator of blood platelets (4, 5) and possesses a variety of actions upon inflammatory cells and the constituent cells of the blood vessel wall. Thrombin is chemotactic for monocytes (6) and is mitogenic for lymphocytes (7) and for mesenchymal cells including vascular smooth muscle cells (8, 9). Thrombin's effects on the vascular endothelium include stimulating endothelial production of prostacyclin (10), platelet-activating factor (11), plasminogen activator-inhibitor (12), and the potent smooth muscle cell mitogen platelet-derived growth factor (13). Thrombin also causes endothelial cells to express P-selectin on their surfaces (14), an action potentially important in mediating the initial adherence of monocytes and neutrophils to an injured or otherwise abnormal artery. Teleologically, these multiple cell-activating functions of thrombin may be viewed as orchestrating normal responses to vascular injury or wounding, potentially mediating not only hemostatic but also inflammatory and proliferative or reparative responses. It is also tempting to hypothesize that thrombin's actions on cells may contribute to pathologic inflammatory and proliferative cellular events in the blood vessel wall, such as those that occur in atherosclerosis and restenosis. However, while thrombin's role as a critical regulator of platelet function in vivo is well established (15–19), the many other cell activating functions of thrombin have been defined only in vitro and remain of uncertain significance in vivo.

We recently isolated a cDNA encoding a functional thrombin receptor (20). The receptor encoded by this clone appears to mediate thrombin-induced platelet activation (20–23) and can mediate a variety of thrombin responses in cultured fibroblasts and endothelial cells (24–26). As a first step toward defining the role of the thrombin receptor in vivo, we have examined thrombin receptor expression in normal and diseased human blood vessels. We now report that the thrombin receptor is expressed in vivo not only by platelets but also by the endothelium of normal-appearing human arteries. Moreover, in atherosclerotic arteries, the thrombin receptor is widely expressed within atheroma both in fatty streaks and in complex atherosclerotic lesions. These data suggest that the recently cloned thrombin receptor may play a role in mediating normal and pathological responses in the blood vessel wall.

Experimental procedures

In situ hybridization. Abdominal aorta obtained from kidney transplant donors and carotid endarterectomy specimens obtained at surgery were fixed in paraformaldehyde and processed for sectioning as previously described (27). Human peripheral blood monocytes were prepared using Histopaque 1077 (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's instructions, then allowed to adhere to glass slides. Alveolar macrophages were obtained from bronchioalveolar lavage specimens and cytospun onto glass slides. Both preparations were then rinsed free of nonadherent cells and fixed for 10 min at 4°C in 4% paraformaldehyde (27) then stored in 70% ethanol/30% water. Sense and antisense ^{35}S-labeled riboprobes were transcribed from human thrombin receptor cDNA (20) or von Willebrand factor cDNA (American Type Culture Collection, Rockville, MD) in pBluescript SK or KS (Strategene Inc., La Jolla, CA) using T7 RNA polymerase; in situ hybridization was performed using published procedures (27). The results on human tissues shown are representative of those obtained with 13 different specimens.

Immunohistochemistry. Frozen tissue sections were reacted with monoclonal antibody Ham 56 (Dako Corp., Carpinteria, CA) to identify monocytes and macrophages or HHF35 (Enzo Pharmaceuticals,

Address correspondence to Shaun R. Coughlin, Box 0524, University of California, San Francisco, San Francisco, CA 94143.

Received for publication 18 May 1992 and in revised form 29 July 1992.

J. Clin. Invest.

0021-9738/92/10/1614/08 $2.00
Volume 90, October 1992, 1614–1621

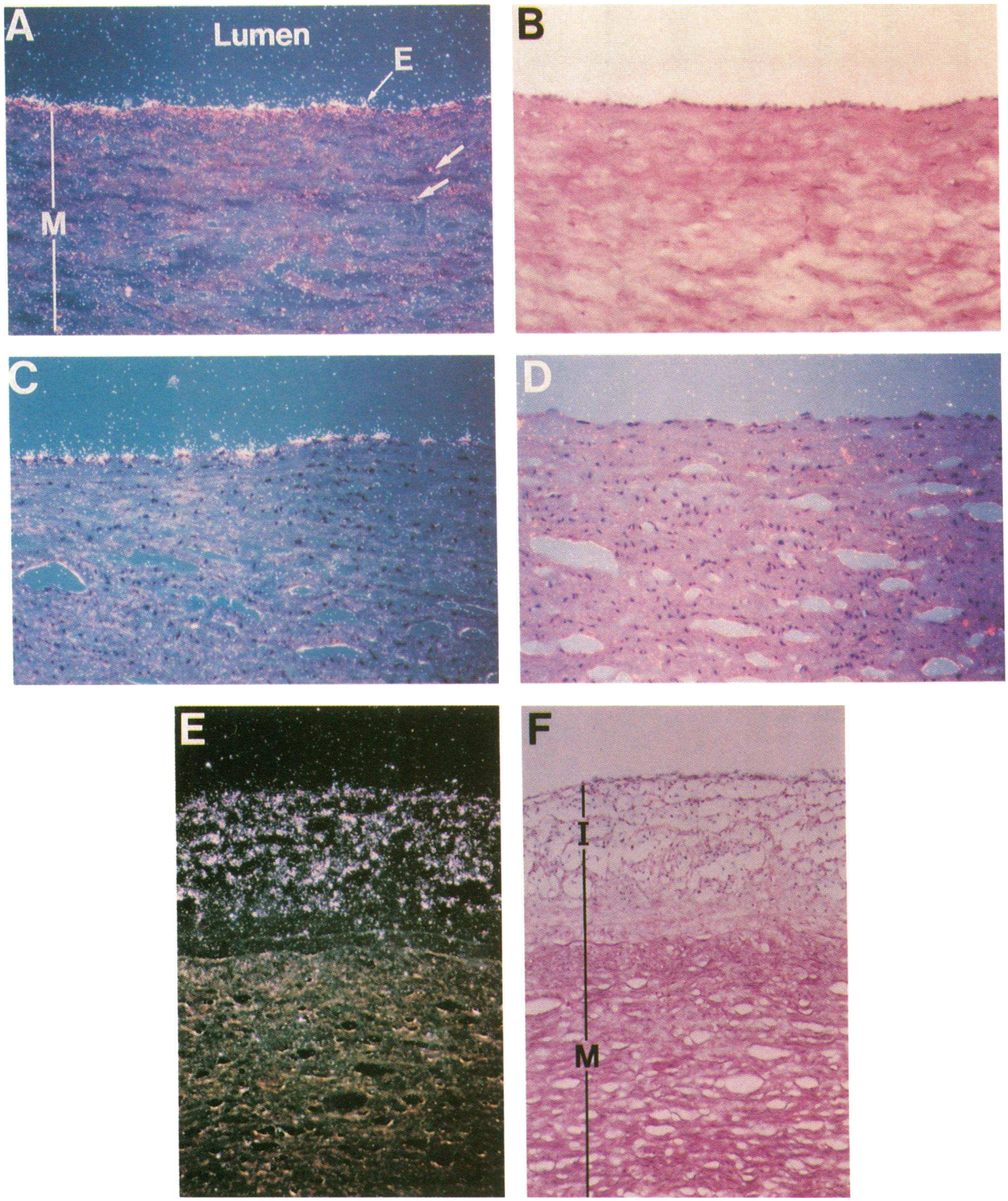
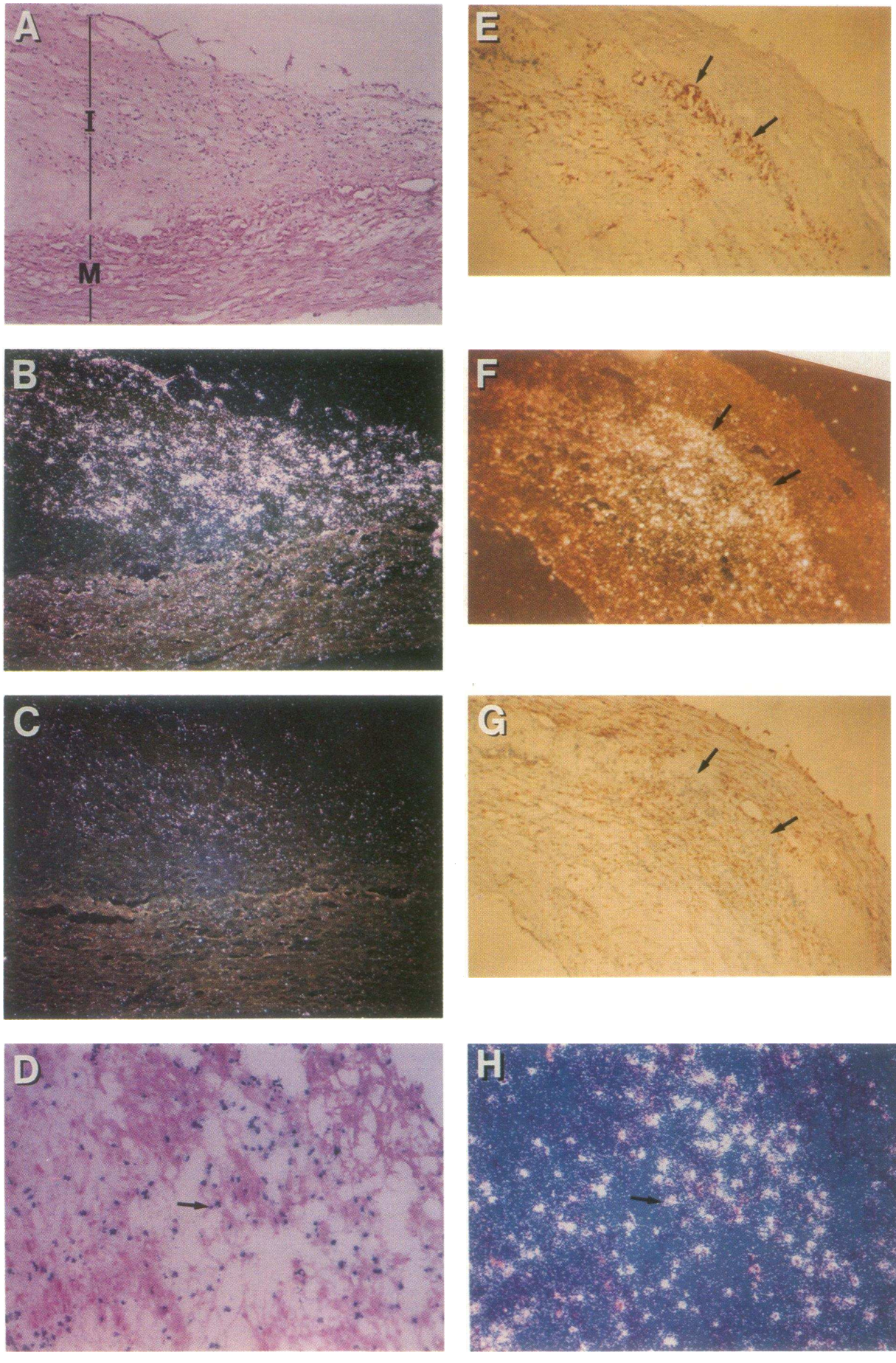


Figure 1. Thrombin receptor expression in normal aorta and fatty streak. In situ hybridization of sections from organ donor aorta for thrombin receptor or von Willebrand factor mRNA. (*A*) Normal-appearing region of aorta revealing positive hybridization for thrombin receptor mRNA expression in the endothelial layer (**E**) (dark-field photomicrograph at ×200; positive hybridization is indicated by the collections of bright grains). Occasional positive cells (*arrows*) were noted in normal-appearing regions of aortic media. (*B*) Bright-field photomicrograph of *A* showing nuclei in the endothelial layer. (*C*) In situ hybridization of a section from the same tissue sample as shown in *A* and *B* for von Willebrand factor mRNA (dark-field photomicrograph at ×200). Note striking collections of silver grains over the endothelial layer. (*D*) Adjacent section to that shown in *C* hybridized with control sense von Willebrand factor probe. Note lack of cell-associated collections of silver grains. Similar results were obtained with sense probes derived from thrombin receptor cDNA. (*E*) Fatty streak revealing diffuse expression of thrombin receptor mRNA in the neointima (dark-field photomicrograph at ×100). (*F*) Same section as *E* photographed in bright-field showing demarcation between the thickened intima (*I*) and underlying media (**M**). Sections were hybridized with antisense or sense ^{35}S-labeled RNA probe transcribed from human thrombin receptor or von Willebrand factor cDNA as described in *Experimental procedures*.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New York) to identify vascular smooth muscle cells as described (27). Thrombin receptor immunohistochemistry was performed using IgG purified from peptide antiserum 1047 which recognizes the human thrombin receptor's hirudin-like domain (23). The IgG as used at a final concentration of 30 μg/ml, the same concentration required to block platelet activation by thrombin (23). Human thrombin receptor–transfected and naive Rat 1 cells as well as incubations without primary antibody or with irrelevant primary antibodies were used to confirm the specificity of thrombin receptor staining. Primary antibodies were detected using biotin-conjugated second antibody and avidin-alkaline phosphatase or avidin-horse radish peroxidase (Vector Laboratories, Burlingame, CA) as described (27).

Cell culture and Northern blot analysis. Human vascular smooth muscle cells were explanted from aorta obtained from transplant donors (28). Human umbilical venous endothelial cells were a generous gift from Dr. Robert Taylor (University of California, San Francisco). Total cellular RNA was prepared using guanidinium thiocyanate lysis and centrifugation through cesium chloride (29). Smooth muscle or endothelial cell RNA was sized by glyoxal gel electrophoresis and analyzed for human thrombin receptor mRNA by Northern blot (29).

Results and Discussion

In situ hybridization of normal-appearing regions of human aorta revealed thrombin receptor mRNA expression in the endothelial layer (Fig. 1 *A*; see also Fig. 2 *F*, endothelial layer at lower left). Only occasional cells in the aortic media showed hybridization for receptor mRNA (Fig. 1 *A*). The presence of endothelial cells in this sample is apparent by hematoxylin-eosin staining (Fig. 1 *B*) and by in situ hybridization for von Willebrand factor mRNA, an endothelial marker (Fig. 1 *C*). The lack of hybridization to the endothelial layer noted when control "sense" probes were used (Figs. 1 *D*, 2 *B* vs. 2 *C*, and data not shown) and the lack of silver grains at the adventitial edge and other tissue boundaries (Fig. 2, *B-lower right* and *F-upper right*) suggested that the endothelial hybridization seen in these studies was specific and did not represent an "edge effect." These data suggest that the thrombin receptor can be expressed by endothelial cells in normal-appearing human arteries. No conclusions regarding the activation state of the endothelium in these samples or any activation dependence of receptor mRNA expression should be drawn from these studies of human pathology specimens. However, we have noted robust thrombin receptor mRNA expression in cardiac and aortic endothelium in mouse embryos that were rapidly fixed (S. J. Soifer, J. O'Keefe, and S. R. Coughlin, manuscript in preparation), again suggesting that normal endothelium can express the receptor in vivo.

In contrast to the localized expression seen in the normal artery, thrombin receptor mRNA was widely expressed in atherosclerotic vessels. In fatty streaks, the early lesions of atherosclerosis, thrombin receptor was widely expressed in the neointima (Fig. 1, *E* and *F*). Little expression was noted in the underlying normal media. In complex atherosclerotic lesions obtained as carotid endarterectomy specimens (Fig. 2, *A–C*), abundant thrombin receptor expression was also noted. Again little receptor expression was seen in the underlying medial layer.

To correlate thrombin receptor expression with the presence of particular cell types within the atheroma, sections adjacent to those probed for thrombin receptor were stained with immunohistochemical markers for monocyte/macrophages or smooth muscle cells using monoclonal antibodies HAM56 or HHF35, respectively. Two distinct patterns of thrombin receptor expression were noted. In the first, thrombin receptor expression correlated with areas of high cellularity staining with the macrophage marker HAM 56 (Fig. 2, *E* and *F* vs. *G*). In the second, thrombin receptor expression was noted in areas which failed to stain for macrophages, but which did stain with the smooth muscle marker HHF35. For example, the particular region of atheroma shown in Fig. 2, *D* and *H*, did not stain with

Figure 2. Thrombin receptor expression in complex plaques: analysis of carotid endarterectomy specimens.

Adjacent sections from a carotid endarterectomy specimen hybridized with antisense thrombin receptor probe to detect receptor mRNA, with sense thrombin receptor probe as a negative control, or were processed for immunohistochemistry using the monocyte/macrophage marker HAM56 or the smooth muscle marker HHF35. (*A*) Carotid plaque. Bright-field photomicrograph (×100) of hematoxylin and eosin–stained section of a complex carotid plaque showing neointima (**I**) and underlying media (**M**). (*B*) Thrombin receptor mRNA in carotid plaque. Dark-field photomicrograph of the same field shown in *A*. Section was hybridized with antisense probe for human thrombin receptor. Exposure time was 10 d. Collections of bright silver grains indicate cells hybridizing for receptor mRNA. (*C*) Sense probe control. Dark-field photomicrograph of a section adjacent to that shown in *B*. This section was hybridized with sense probe for thrombin receptor mRNA and exposed and developed in parallel with that shown in *B*. Note absence of hybridizing cells (compare to *B*). (*D*) Macrophage-poor region of atheroma. Bright-field photomicrograph (×200) of a region of complex plaque containing HHF35 positive (smooth muscle) and nonstaining mesenchymal-appearing intimal cells but no HAM56 positive (monocyte/macrophage) cells. The section was stained with hematoxylin and eosin stain to show nuclei for comparison with the dark-field photomicrograph of the same field showing thrombin receptor hybridization (*H*); arrows show the same cell in each photo for orientation. (*E*) Macrophage immunostaining of carotid plaque. Bright-field photomicrograph (×50) of a section of carotid plaque immunostained with monocyte macrophage marker HAM56. Positive staining (*arrows*) is seen as the brown peroxidase product. For orientation, the endothelial surface is lower left, medial layer is upper right. (*F*) Dark-field photomicrograph (×50) of a carotid plaque section adjacent to that shown in *E* hybridized for thrombin receptor mRNA. Positive hybridization denoted by collections of silver grains. Note band of positive cells (*arrows*) that correlates with area of HAM56 staining for monocyte/macrophages. Note also the lack of hybridization to the medial layer seen at upper right; also seen in *G* as the layer at the upper right with linear HHF35 staining. Lastly, note the lack of silver grains at the adventitial outer edge (*extreme upper right*) suggesting that the silver grains overlying the endothelial layer (*lower left*) represent hybridization and not "edge effect" (see also Fig. 1). (*G*) Smooth muscle immunostaining of carotid plaque. Bright-field photomicrograph (×50) of section adjacent to *F* stained with the smooth muscle marker HHF35. Positive staining is seen as the brown peroxidase product. Arrows show the cellular area corresponding to that staining for macrophages in *E*. (*H*) Macrophage poor region of atheroma shows thrombin receptor hybridization. Dark-field photomicrograph corresponding to the bright-field view shown in *D*. Silver grains indicate hybridization for thrombin receptor mRNA to nearly all cells in this field (compare nuclei in *D* to collections of silver grains in *H*). Immunostaining of adjacent sections revealed no macrophage staining in this region. Approximately half of the cells in this region did stain with the smooth muscle cell marker HHF35 and half remained unstained, the latter presumably representing mesenchymal-appearing intimal cells.

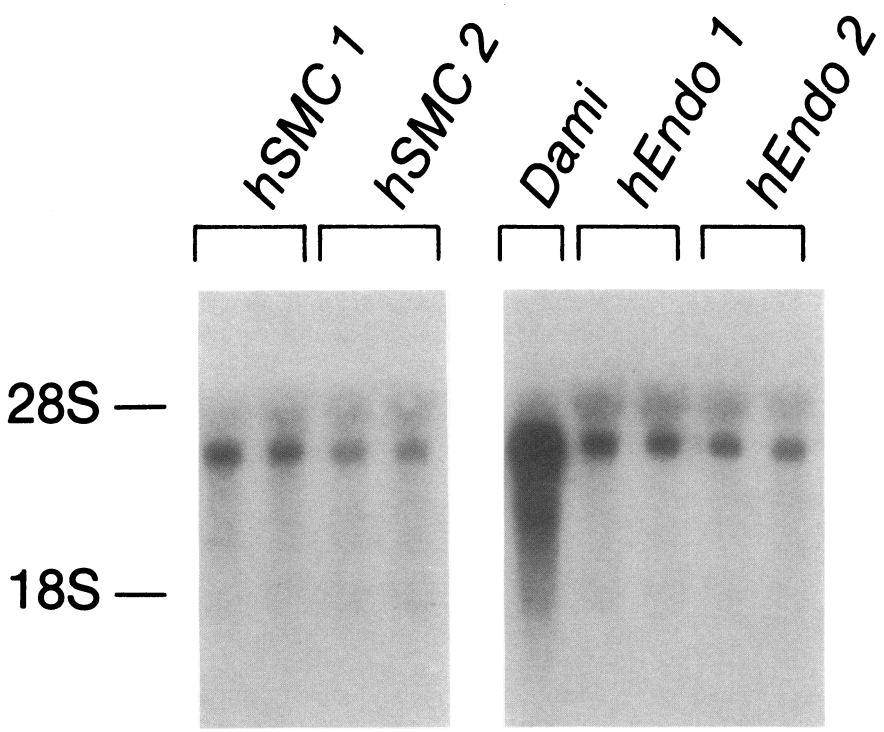

Figure 3. Northern blot analysis of thrombin receptor mRNA expression by vascular cells in culture. Total cellular RNA was prepared from cultured human aorta–derived smooth muscle cells and from human umbilical venous endothelial cells and was analyzed by Northern blot for thrombin receptor mRNA. (*hSMC*) 8 μg total cellular RNA from two separate isolates of human smooth muscle cells. (*hEndo*) 8 μg of total cellular RNA from two separate isolates of human umbilical venous endothelial cells. (*Dami*) 8 μg polyA+ RNA from Dami cells included as a positive control. RNA size standards are indicated at left.

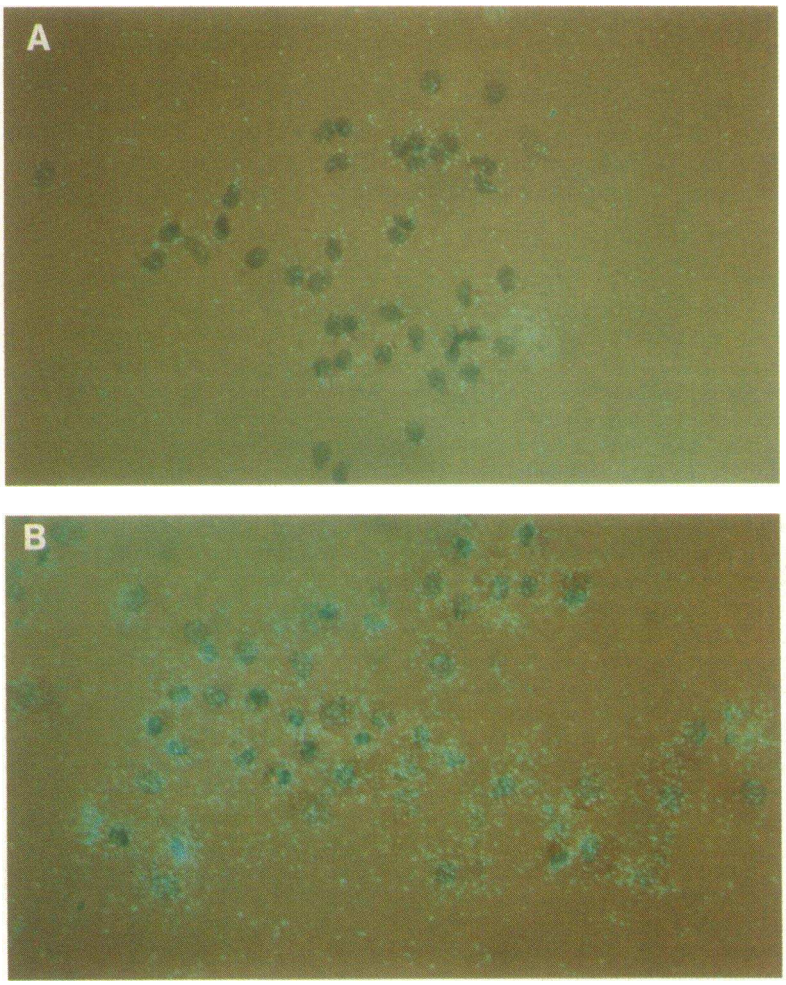

Figure 4. In situ hybridization of peripheral blood monocytes and bronchioalveolar lavage-derived macrophages for thrombin receptor mRNA. Epiluminescence photomicrograph (×400). Bright blue dots indicate silver grains denoting the presence of thrombin receptor mRNA. Cells were fixed and hybridized for thrombin receptor mRNA as described in *Experimental procedures.* Greater than 90% of cells in the peripheral blood monocytes and bronchioalveolar lavage preparations had the morphologic features of monocytes or macrophages, respectively, by Wright's stain. Even at 8 wk exposure, only a very low signal for thrombin receptor mRNA was detected in monocytes (*A*). By contrast, at 4 wk exposure, clear hybridization was noted in macrophages (*B*). The sense probe controls for both monocytes and macrophages revealed no positive hybridization. Expression of thrombin receptor protein in macrophages was confirmed by immunohistochemical staining (not shown).

the macrophage marker HAM56, while other regions of this same slide did stain. In this HAM56 negative region, ~ 50% of the cells did stain with the smooth muscle marker HHF35. The remainder of cells in this region did not stain with either HHF35 or HAM56 and appeared to represent "mesenchymal-appearing intimal cells" (27, 30, 31). The latter cells are not recognized by available cell-specific antibodies, but may represent "synthetic state" smooth muscle cells. Note that nearly all cells in this particular field did express thrombin receptor mRNA (Fig. 2, *D* and *H*), suggesting that both smooth muscle and mesenchymal-appearing intimal cells express receptor.

Expression of thrombin receptor mRNA by vascular endothelial and smooth muscle cells was confirmed by Northern analysis (Fig. 3). These data coupled with the in situ mRNA localization data cited above suggest that human vascular endothelial cells and plaque smooth muscle cells can indeed express the thrombin receptor in vivo. Thrombin receptor mRNA expression in peripheral blood monocytes was difficult to detect by Northern analysis. For this reason, and because contamination of preparations of monocytes with other cell types would confound interpretation of any low level of receptor expression found with more sensitive techniques, thrombin receptor ex-

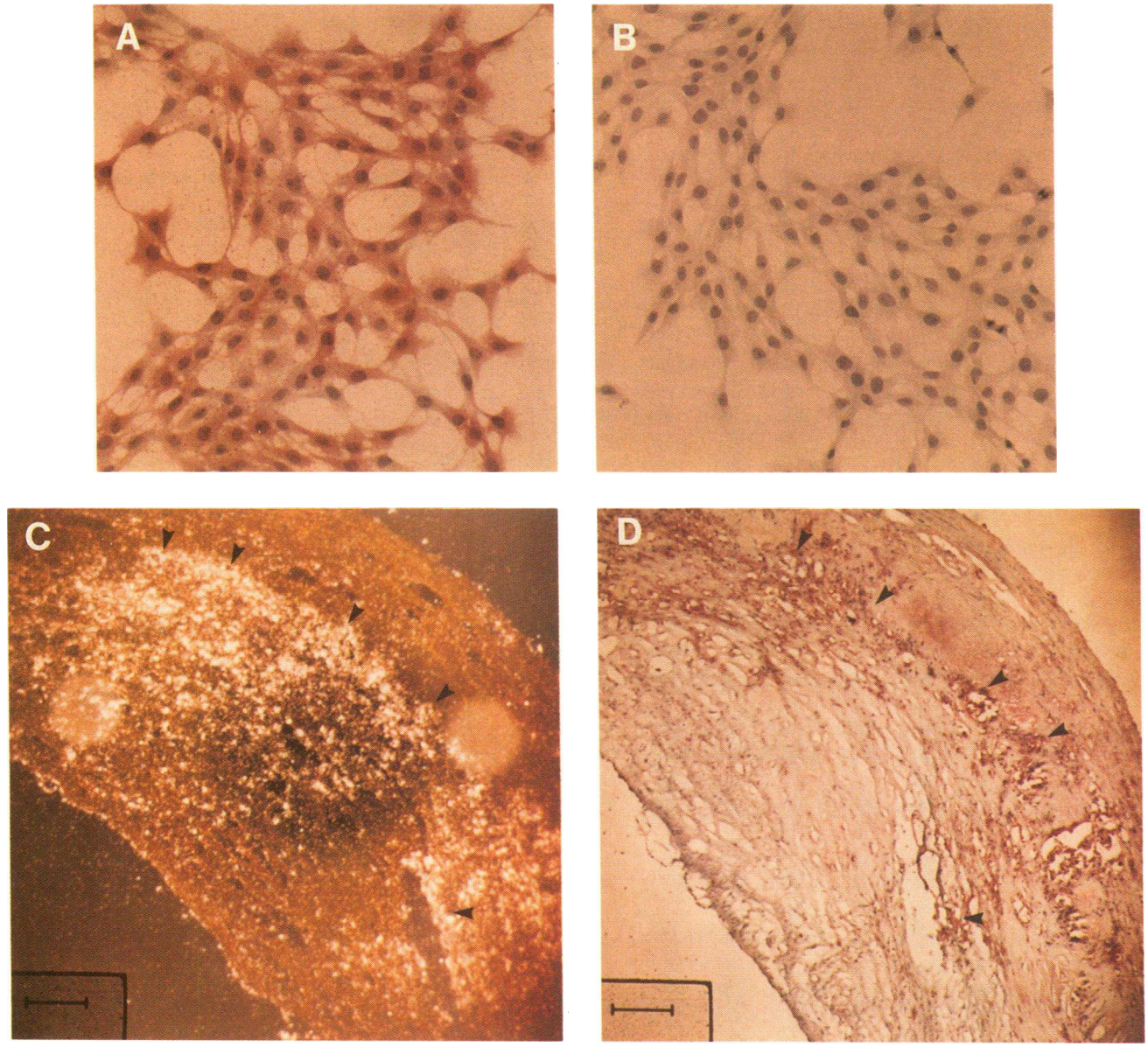

Figure 5. Immunohistichemistry for thrombin receptor protein in receptor transfected cells and naive cells (*A* and *B*) and in human atheroma (*D*); correlation with in situ hybridization (*C*). Immunostaining is seen as the dark red alkaline phosphatase product. Cells or tissues were incubated with IgG purified from a rabbit peptide antisera to human thrombin receptor (23) and processed as described in *Experimental procedures*. (*A* and *B*) Rat 1 cells stably transfected with human thrombin receptor cDNA (*A*) or naive Rat 1 cells (*B*) immunostained for human thrombin receptor (bright-field, ×400). (*C* and *D*) Human carotid endarterectomy specimen. Sections near those shown in Fig. 2, *E–G*, were hybridized for thrombin receptor mRNA (*C*) or immunostained for human thrombin receptor (*D*). Note the lack of immunostaining in control Rat 1 cells (*B*) compared to transfected cells (*A*) and the correlation of areas of immunostaining (*arrows, D*) with areas of positive hybridization for thrombin receptor (*arrows, C*). Scale bars represent 200 μm *C* is a dark-field photomicrograph at ×50. *D* is a bright-field at ×50.

pression in these cells was sought by in situ hybridization of isolated cells. Thrombin receptor expression in isolated peripheral blood monocytes appeared to be present at very low levels; an eight-week exposure was required for the development of the in situ hybridization shown in Fig. 4 *A*. By contrast, expression in isolated macrophages obtained by bronchioalveolar lavage was readily seen after four weeks (Fig. 4 *B*). Immunohistochemical staining with thrombin receptor antibody (see below) also suggested receptor expression by macrophages. These data indicate that human macrophages can express thrombin receptor and coupled with our in situ and immunostaining results (Fig. 2) suggest that macrophages within human atheroma express thrombin receptor mRNA. How thrombin receptor expression might be upregulated in tissue macrophages compared to circulating monocytes, and how much of the intense thrombin receptor expression seen in macrophage-rich areas is contributed by macrophages themselves vs. by target cells responding to macrophage cytokines, remains to be defined.

The above studies demonstrated thrombin receptor expression in vivo at the mRNA level. To confirm thrombin receptor expression at the protein level, we used IgG purified from a thrombin receptor peptide antiserum for immunohistochemical studies. The antiserum recognizes the human thrombin receptor's hirudin-like domain and blocks thrombin-induced platelet activation (23). Rat 1 cells transfected with human thrombin receptor cDNA stained positively with the antiserum but the untransfected parent cell line did not (Fig. 5, *A* vs. *B*), demonstrating the specificity of the antiserum. In human atheroma, positive staining with the receptor antiserum correlated with positive hybridization for thrombin receptor mRNA (Fig. 5, *C* vs. *D*). In more normal vessels, endothelial staining with the thrombin receptor antiserum was detected (not shown). These data suggest that the thrombin receptor is expressed at the protein level in human arteries.

To our knowledge, this is the first study demonstrating thrombin receptor expression in vivo. Given the known activities of thrombin on vascular cells in culture, the demonstration of thrombin receptor expression in the blood vessel wall establishes thrombin receptor activation as a candidate for mediating inflammatory and proliferative events in the artery wall in vivo.

The observation that thrombin receptor mRNA is expressed by arterial endothelium suggests that these cells are capable of responding to thrombin in vivo. Some or all of the host of thrombin responses described for cultured endothelial cells (see Introduction) may thus be elicited in vivo via the cloned thrombin receptor and modulate thrombotic, inflammatory, and proliferative responses in the vessel wall.

Thrombin receptor expression by macrophages is tantalizing in that these cells are known to express tissue factor (31). The latter observation predicts that macrophages exposed to circulating coagulation factors would trigger local thrombin production, a situation that may occur in a plaque with altered endothelial permeability. Whether locally produced thrombin plays a role in localizing or activating macrophages remains to be seen.

The observation that plaque smooth muscle cells express thrombin receptor mRNA suggests that these cells may also respond to thrombin in vivo, and prompts hypotheses regarding possible roles for thrombin in stimulating smooth muscle cell proliferation in vivo. Such stimulation could occur directly upon receptor activation or indirectly by induction of autocrine or paracrine growth factors (9, 13, 28, 30). Potential sources of thrombin in the plaque include possible local thrombin generation in the absence of frank plaque rupture and thrombosis (see above), as well as thrombin generated within thrombi in ruptured atherosclerotic plaques. Such thrombi are often seen, even in autopsies of patients dying of noncardiac causes, and the hypothesis that organization of plaque thrombi within complex atherosclerotic plaques contributes to disease progression is long extant (reviewed in reference 32). A recent study using an animal model of restenosis following vessel injury does suggest a possible role for thrombin per se in promoting intimal proliferation (33). The possibility that thrombin receptor activation in vivo may contribute to the progression of atherosclerosis or to restenosis following angioplasty by promoting cell proliferation and organization of plaque thrombi remains to be tested.

Acknowledgments

This work was supported in part by National Institutes of Health (NIH) grants HL-43322, HL-44907, and HL 43821, and by University of California Tobacco-Related Disease Research Program grant No. 2RT-19, and was done during the tenure of an award to S. R. Coughlin from the American Heart Association and SmithKline Beecham. N. Nelken and J. O'Keefe were supported in part by the Pacific Vascular Foundation. S. Soifer was supported by NIH grant HL-35518. I. Charo was supported in part by NIH grant HL-42662. We are grateful to Drs. J. Rapp and Jerry Goldstone, and to University of California, San Francisco's Department of Pathology for vascular tissues.

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