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Disparate Temporal Expression of the Prothrombin and Thrombin Receptor Genes during Mouse Development

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The protease thrombin is a potent agonist for platelet aggregation, mesenchymal cell proliferation, and endothelial production of growth factors and adhesion molecules. Thrombin also modulates neurite outgrowth in neuronal cultures. These apparently disparate responses to thrombin appear to be largely mediated by the recently cloned thrombin receptor. In the adult, thrombin is generated from its zymogen prothrombin at sites of vascular injury when circulating coagulation factors meet extravascular tissue factor. In this context thrombin's varied actions may mediate responses to wounding. Whether thrombin's actions on cells may also play a role in development is unknown. We examined the expression of thrombin receptor, prothrombin, and tissue factor by in situ hybridization in mouse development. Thrombin receptor mRNA was expressed widely in mesenchymal cell populations during early organogenesis (E9.5) and was particularly abundant in developing heart and blood vessels. Robust receptor expression was also noted in the germinal epithelium of the hindbrain. Thrombin receptor expression became more restricted with time and by the fetal growth stage (E16.5) was most readily detected in certain neurons, endocardial and endothelial cells, and within lung and liver. In contrast to the thrombin receptor, prothrombin mRNA was limited to the embryonic liver and was not detected until E12.5, well after the onset of receptor expression. mRNA for tissue factor, one important trigger for thrombin generation in the adult, was detected in embryonic epithelia from E9.5–12.5. In several instances, tissue factor-expressing epithelia were surrounded by thrombin receptor-expressing mesenchyme. These data suggest a possible role for the thrombin receptor in development. The finding of robust thrombin receptor expression before prothrombin mRNA was detected raises the question of whether other proteases or peptide ligands can activate the thrombin receptor. (Am J Pathol 1994, 144:60–69)

Thrombin, a multifunctional serine protease, is a potent agonist for a variety of cellular functions.[1] *In vitro*, thrombin is the most potent activator of blood platelets,[2] is chemotactic for monocytes,[3] and is mitogenic for fibroblasts and vascular smooth muscle cells.[4,5] Thrombin acts on vascular endothelial cells to stimulate production of platelet-derived growth factor and other bioactive molecules.[6–9] In neuronal cultures, thrombin modulates neurite outgrowth.[10]

In the adult, thrombin is generated at sites of vascular injury. Cleavage of the inactive zymogen prothrombin is triggered when circulating coagulation factors meet extravascular tissue factor[11] and by tissue factor-independent pathway(s).[12] In this context, thrombin's actions on a variety of cell types may function to orchestrate hemostatic, inflammatory, and proliferative or reparative responses to wounding. Whether thrombin's varied mitogenic and metabolic actions also govern cellular behavior during development is unknown.

Many of thrombin's cell signaling activities appear to be mediated by the recently cloned thrombin receptor.[5,13–17] To gain insight into the possible role of the thrombin/thrombin receptor system in mamma-

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lian development, we examined the expression of prothrombin, tissue factor, and the thrombin receptor during organogenesis (E9.5-E16.5) in mouse embryos using *in situ* hybridization.

Materials and Methods

Tissue Samples

Swiss-Webster mouse embryos (9.5, 10.5, 11.5, 12.5, 14.5, and 16.5 days post-coitus; p.c.) were fixed in 4% paraformaldehyde in phosphate-buffered saline overnight. After fixation, the embryos were dehydrated in ethanol, cleared in toluene, and embedded in paraffin. Ten-micron sections from the paraffin embedded embryos were mounted on aminoalkylsilane-treated slides. The slides were stored at 4 C until used.

Probe Generation

Plasmids for transcribing two independent probes for thrombin receptor mRNA were generated by subcloning nonoverlapping 0.3-kb *Apa*I-*Acc*I and 0.7-kb *Pst*I to *Xho*I fragments of murine thrombin receptor cDNA[5,18] into pBluescript SK. Identical expression patterns were seen with these two independent probes; the results shown were obtained with the 0.7-kb fragment. Murine prothrombin and tissue factor cDNAs were cloned by polymerase chain reactions using primers based on published sequences.[19,20] A 1.0-kb *Xho*I-*Pst*I prothrombin cDNA fragment and a 0.4-kb *Xba*I-*Eco*RV tissue factor cDNA fragment were also each subcloned into pBluescript SK. Plasmids were linearized with appropriate restriction enzymes and transcribed using T3 or T7 RNA polymerases (Boehringer-Mannheim, Indianapolis, IN) with ^{35}S-labeled UTP (New England Nuclear, Boston, MA) to generate antisense or sense radiolabeled RNA probes.

In Situ Hybridization

In situ hybridization was performed using a modification of published procedures.[21–23] Tissues sections were deparaffinized, fixed in 4% paraformaldehyde in phosphate-buffered saline, treated with proteinase K, and acetylated (0.25% acetic anhydride in 0.1 mol/L triethanolamine-HCl, pH 7.5). After washing in 0.5X SSC, the sections were covered with hybridization solution (50% deionized formamide, 0.3 mol/L NaCl, 20 mmol/L Tris pH 8, 5 mmol/L EDTA, 1X Denhardt's, 10% dextran sulfate, and 10 mmol/L dithiothreitol) and prehybridized for 1 to 3 hours at 55 C. ^{35}S-labeled antisense RNA probes (600,000 cpm/slide) were added to the hybridization solution and the incubation continued for 12 to 18 hours at 55 C. To control for nonspecific "hybridization," serial sections were processed in parallel with the corresponding sense probes. After hybridization, the sections were washed for 20 minutes in 2X SSC, 10 mmol/L β-mercaptoethanol, and 1 mmol/L EDTA, treated with RNAse A (20 μg/ml) for 30 minutes at room temperature, then washed at high stringency (0.1X SSC, 10 mmol/L β-mercaptoethanol, and 1 mmol/L EDTA) for 2 hours at 55 C. The sections were dehydrated, dipped in photographic emulsion (Ilford), stored at 4 C, developed after 2 to 10 weeks of exposure, and counterstained with hematoxylin and eosin.

Limitation of in Situ *Hybridization Studies*

These studies define the expression of mRNAs encoding three key proteins in the thrombin/thrombin receptor system; they do not examine the levels or activities of the proteins themselves. The ability to determine thrombin receptor antigen levels in the mouse awaits the development of appropriate antibodies. Our limited experience comparing *in situ* hybridization versus immunohistochemical determination of human thrombin receptor expression suggests the receptor protein is expressed at levels equal to or greater than predicted by mRNA levels (ref. 21 and data not shown).

Results and Discussion

To localize embryonic expression of thrombin receptor, prothrombin, and tissue factor, sections of mouse embryos from E9.5 through E16.5 were hybridized with ^{35}S-labeled antisense transcripts and processed as described in Materials and Methods. In general, thrombin receptor mRNA was broadly distributed in embryonic mesenchymal tissues during the early stages of organogenesis (E9.5-E12.5), but became more restricted in later development (Figure 1, A-D). By the fetal growth stage (E16.5) receptor mRNA was most easily detected in certain neurons, in endocardial and endothelial cells, in lung parenchyma, and in liver (Figure 1D and not shown). At this stage of development, liver is a prominent site of hematopoiesis, and receptor mRNA was most abundant in very large cells, pre-

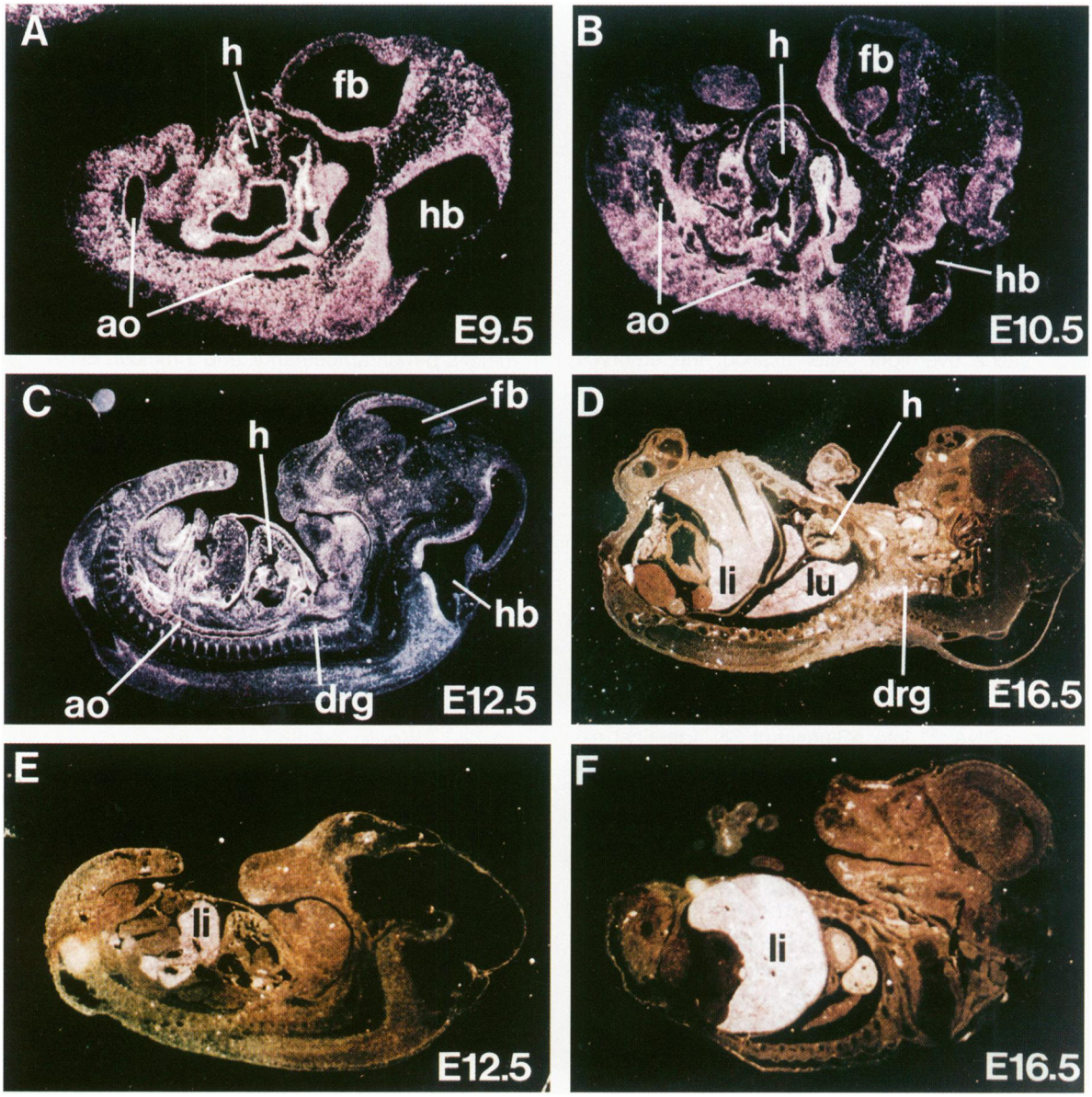

Figure 1. *Thrombin receptor and prothrombin expression in the developing embryo. Sagittal embryo sections at 9.5, 10.5, 12.5, and 16.5 days p.c. were hybridized with antisense probes for thrombin receptor* (**A-D**) *or prothrombin* (**E, F**). *ao, aorta; h, heart; fb, forebrain; hb, hindbrain; drg, dorsal root ganglia; li, liver. Dark-field; magnification* ×*65* (**A**); ×*50* (**B**); ×*20* (**C**); ×*10* (**D**); ×*25* (**E**); ×*10* (**F**).

sumably megakaryocytes. In contrast to thrombin receptor mRNA, hybridization for prothrombin mRNA was restricted to liver and curiously was not expressed until well after the receptor mRNA appeared (Figure 1, E and F). Hybridization for tissue factor mRNA was also quite restricted. Comparison of the spatial and temporal expression patterns of these components of the thrombin/thrombin receptor system raises a number of interesting questions, as discussed below.

Thrombin Receptor mRNA Expression in the Embryonic Heart and Blood Vessels

Thrombin receptor mRNA was particularly abundant in the developing heart and blood vessels (Figures 2 and 3). In the embryonic heart, thrombin receptor mRNA was most strikingly localized in the endocardium and also in mesenchymal cells in the endocardial cushions (Figures 1 and 2). This pattern of expression suggests a possible role for the thrombin

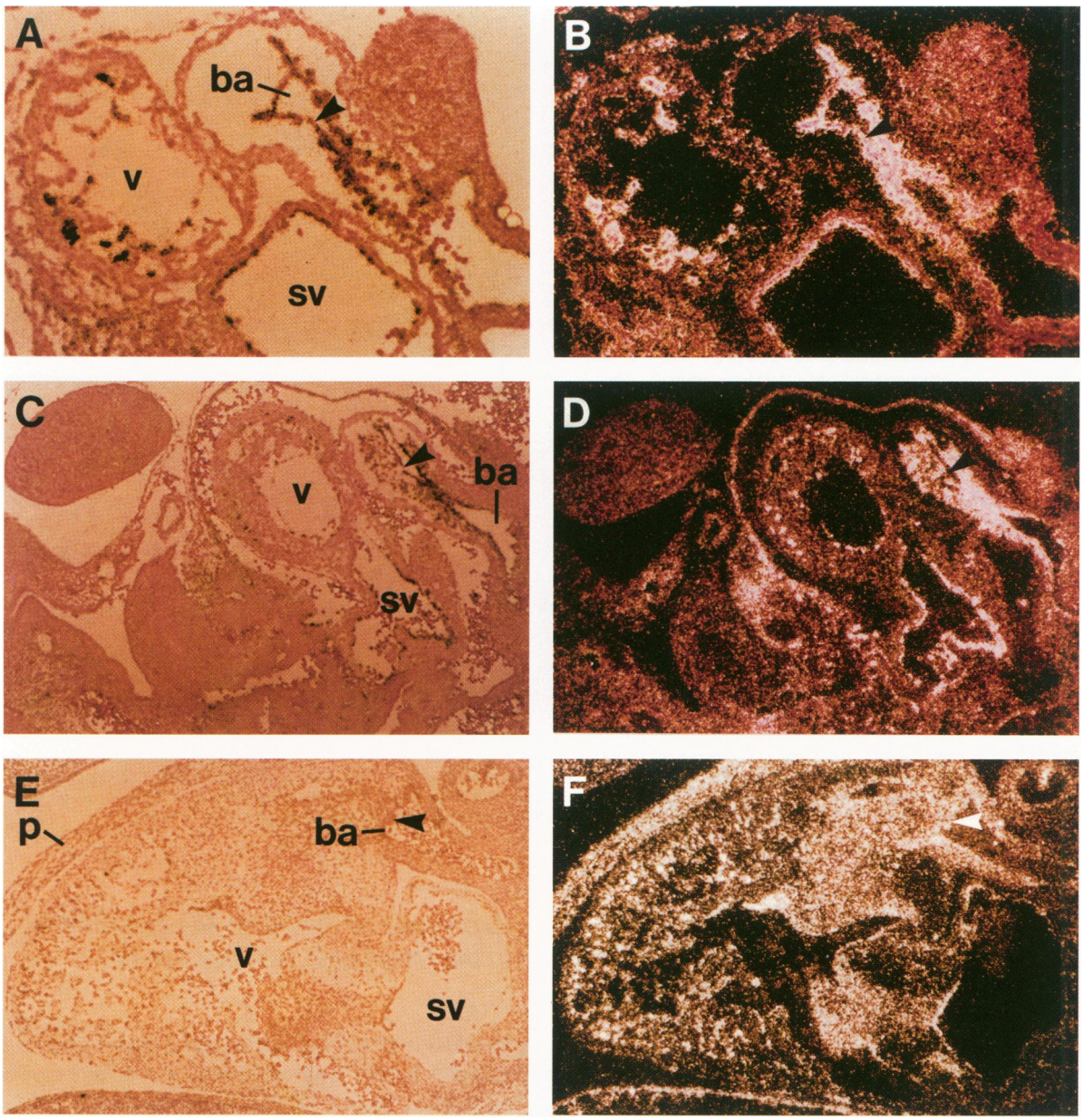

Figure 2. *Thrombin receptor expression in the developing heart. Sagittal embryo sections at 9.5* (**A, B**), *10.5* (**C, D**) *and 12.5* (**E, F**) *days p.c. were hybridized with antisense probe for thrombin receptor. Arrows indicates endothelial cells overlying the endocardial cushions. sv, sinus venosus; v, ventricle; ba, bulbus arteriosus; p, pericardium.* **B, D,** *and* **F** *are dark-field views of* **A, C,** *and* **E,** *respectively. Magnification* ×*200* (**A, B**) *or* ×*100* (**C–F**).

receptor in the complex morphogenetic events that drive septation and cardiac valve formation. Other growth factors and their receptors including members of the transforming growth factor-β family and the FGF receptor family are also expressed in the endocardial cushions of the embryonic heart.[22,24] Thrombin's ability to synergize with fibroblast growth factor to effect cell proliferation[25] suggests possible interactions among these growth factor families during heart development.

Beyond the endocardium, thrombin receptor mRNA was detected in the endothelium of both large and small blood vessels during organogenesis; examples of positive hybridization to aorta and to a small vessel supplying the cranial mesenchyme are shown (Figures 1, 3, and 4). The expression of

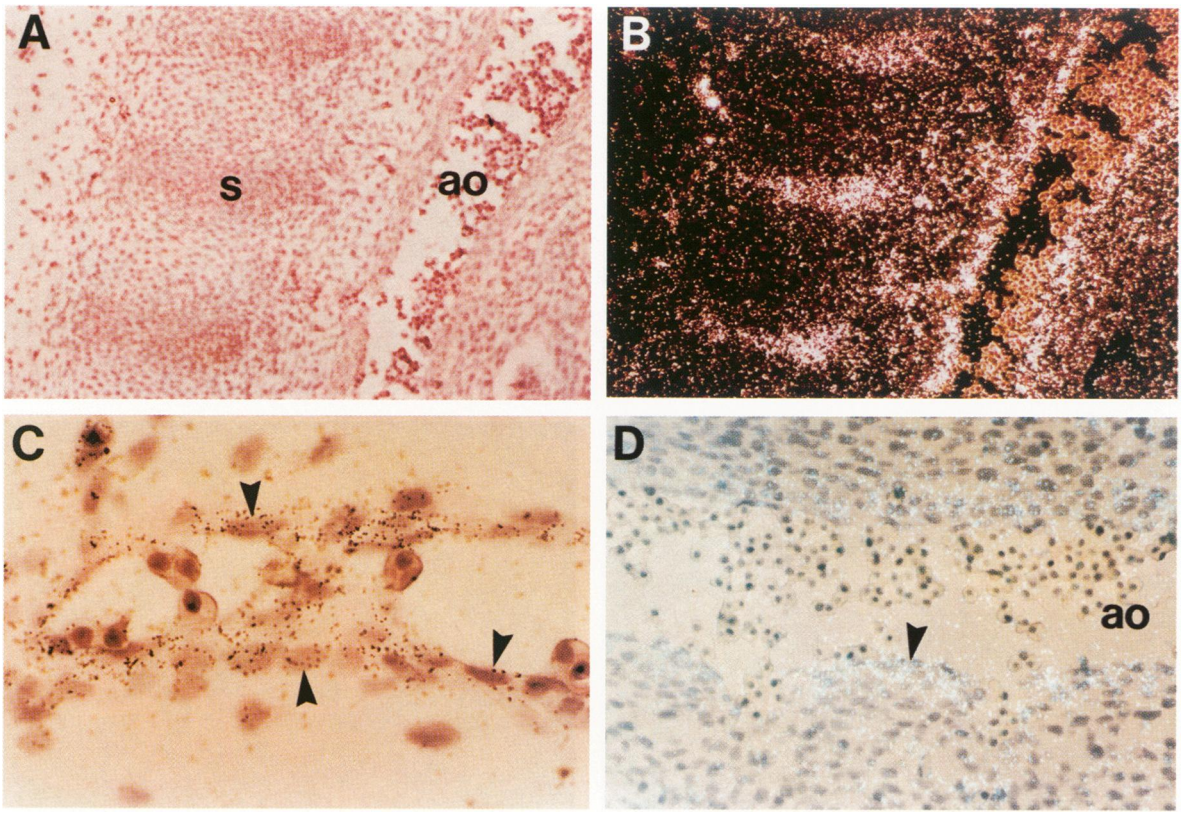

Figure 3. *Thrombin receptor expression in developing blood vessels. Embryo sections at 12.5 days p.c. were hybridized with antisense probe for thrombin receptor.* A *and* B *are paired bright and dark-field vies of aorta (magnification ×200; ao, aorta; s, somites). Arrows indicate endothelial cells.* D *shows aorta at ×400 photographed using epi-illumination to visualize the silver grains and transmitted light to visualize the tissue.* C *depicts a small artery in the brain (×1000); arrows show cells with collections of silver grains indicating thrombin receptor mRNA.*

thrombin receptor mRNA in embryonic endothelium and thrombin's known ability to induce growth factor production by endothelial cells[6] suggests a possible role for thrombin in blood vessel growth and development.

Expression of thrombin receptor mRNA was also detected in cells deep to the endothelium in the aorta at E12.5, suggesting expression in embryonic smooth muscle cells (Figures 2 and 3A-B and D). Interestingly, in adult human aorta, thrombin receptor mRNA was difficult to detect in vascular smooth muscle cells in the normal vessel wall but was robustly expressed in atherosclerotic plaques by several cell types including smooth muscle.[21] The finding of thrombin receptor expression in the developing vessel wall and in atherosclerotic plaque suggests that the thrombin receptor may play a role in controlling smooth muscle cell proliferation and/or differentiation. Alternatively, receptor expression may be a marker for these processes.

Expression of Thrombin Receptor mRNA in the Embryonic Nervous System

In addition to the cardiovascular system, thrombin receptor mRNA was expressed in the embryonic nervous system. During organogenesis (E9.5-E12.5) thrombin receptor RNA was detected in the germinal epithelium of the hindbrain (Figures 1 and 4A-B). Later (E12.5-E16.5), relatively high levels of thrombin receptor mRNA were found in neurons in the ganglia of cranial nerves V, VIII, and X and the dorsal root ganglia (Figures 1 and 4C-F). In the adult nervous system, thrombin receptor mRNA was also expressed by neurons in hindbrain nuclei and in the Purkinje cells of the cerebellum (data not shown). Thrombin, in contrast to other growth factors, inhibits neurite outgrowth and causes neurite retraction, apparently via activation of the cloned receptor.[10] The widespread early expression of

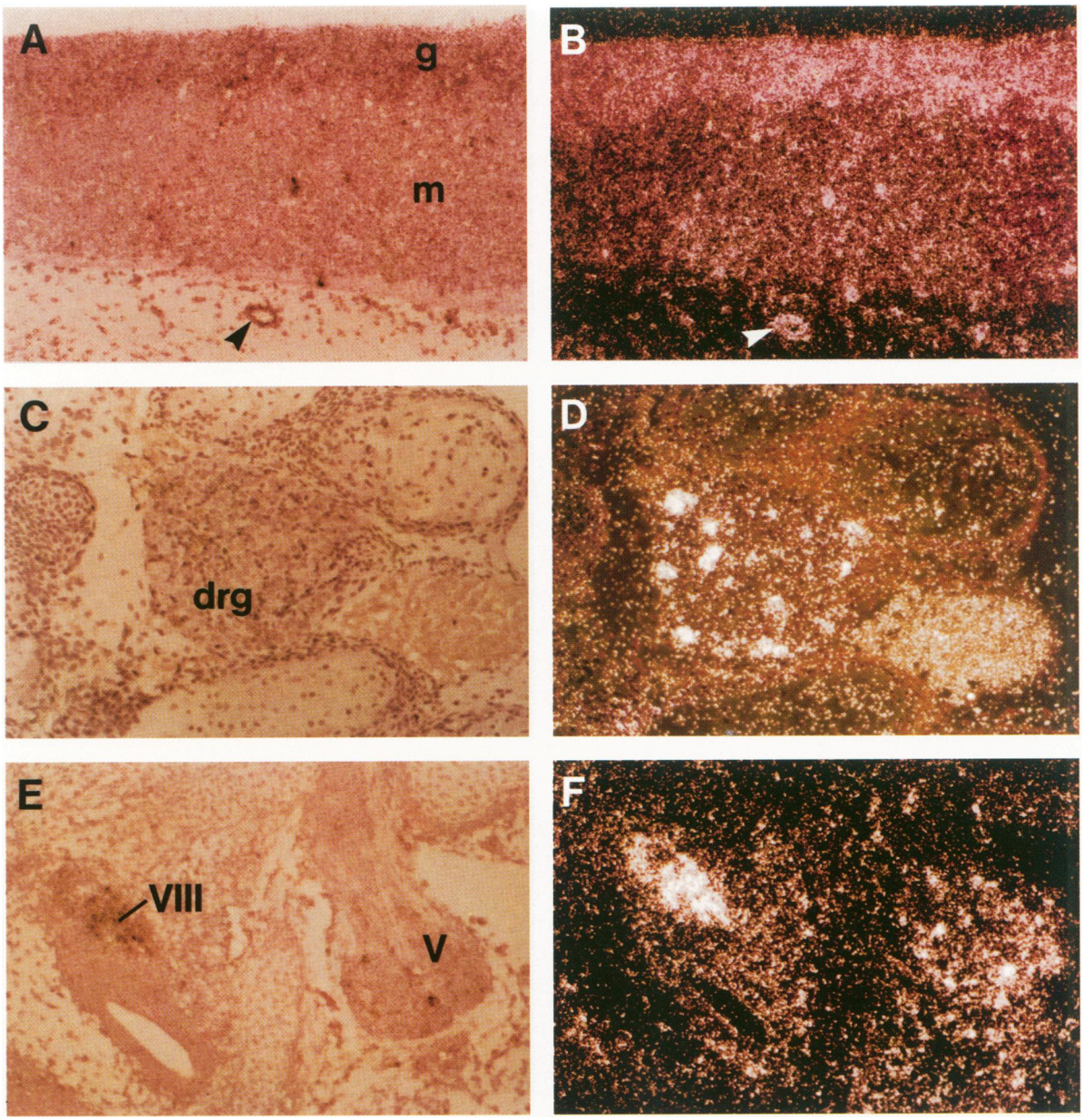

Figure 4. *Thrombin receptor expression in the developing nervous system. Embryo sections at 11.5* (**A, B**) *and 14.5* (**C-F**) *days p.c. were hybridized with antisense probes for thrombin receptor. Arrow indicates a small sized artery. g, germinal epithelium of the myelencephalon, drg, dorsal root ganglion, V, Vth cranial nerve nucleus, VIII, VIIIth cranial nerve nucleus. Paired bright and dark-field photos* (**A, C,** *and* **E** *versus* **B, D,** *and* **F,** *respectively) are shown; magnification ×200.*

thrombin receptor mRNA in the embryonic nervous system suggests a possible novel role for thrombin in limiting dendrite outgrowth during cell proliferation and/or in the extensive remodelling of neural processes that occurs during development. The role of the more focal and persistent thrombin receptor expression in neurons later in development and in the adult is unknown.

Expression of Tissue Factor mRNA in Embryonic Epithelia

Tissue factor is probably the major trigger for thrombin generation,[11] but tissue factor-independent mechanisms exist.[12] From E9.5 to E12.5 tissue factor mRNA was robustly expressed in corneal and

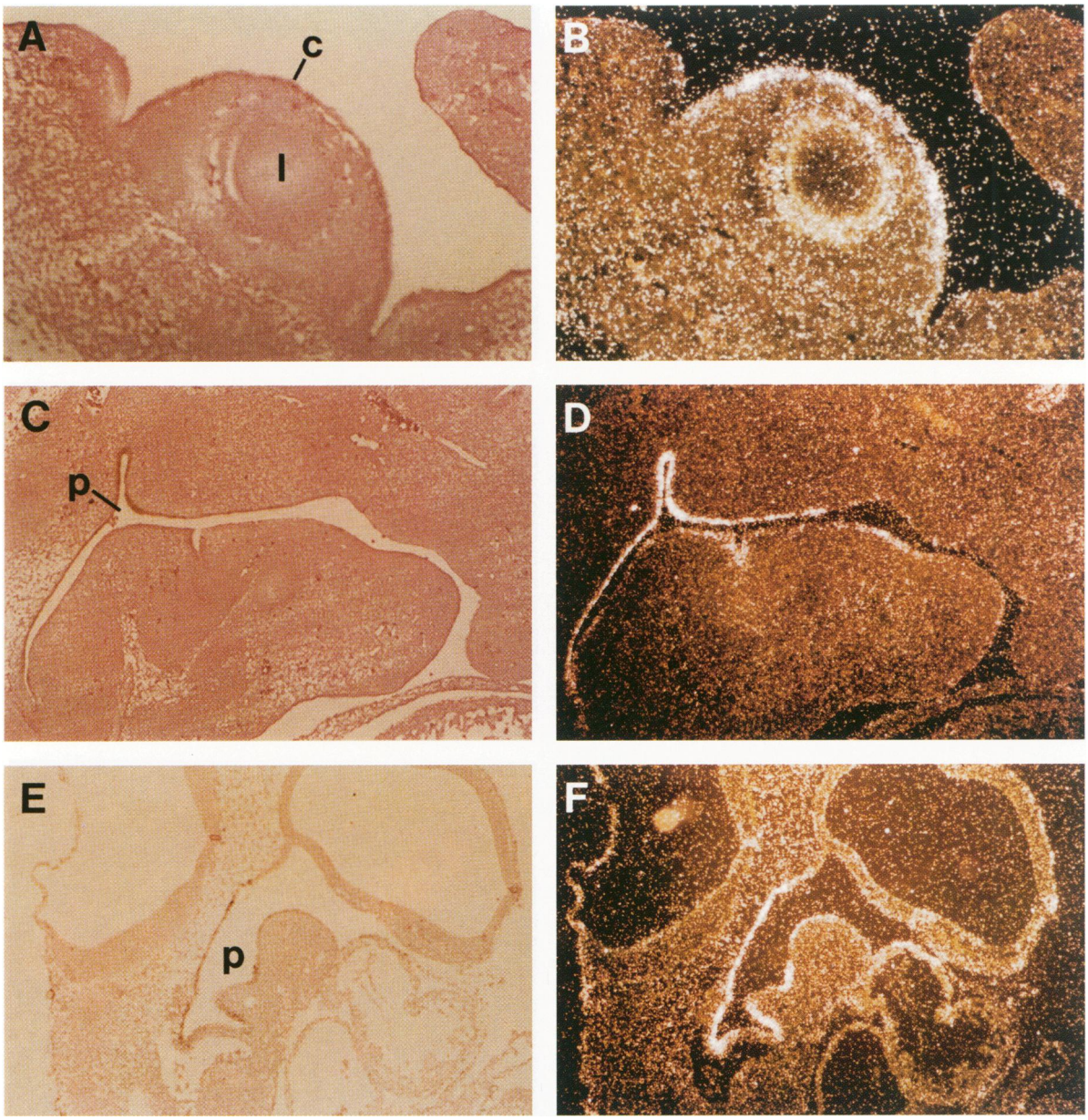

Figure 5. *Tissue factor expression in developing epithelium. Embryo sections at 9.5* (E, F) *and 12.5* (**A-D**) *days p.c. were hybridized with antisense probe for tissue factor. Paired bright and dark-field photos* (**A, C,** *and* **E** *versus* **B, D,** *and* **F,** *respectively*) *are shown. Magnification* ×*100* (**C-F**) *and* ×*200* (**A, B**). *c, cornea; l, lens; p, pharynx.*

pharyngeal epithelium (Figure 5). Obvious co-localization of thrombin receptor and tissue factor mRNAs was noted in the embryonic lung, gut, and epidermis. In the gut and lung, tissue factor was expressed by the epithelium and the thrombin receptor by the surrounding mesenchyme (Figures 1 and 6 and data not shown). In the embryonic epidermis, tissue factor was expressed by the suprabasal cells and thrombin receptor by the basal cells (Figure 6, E-F). These observations suggest the possible existence of local networks for generating and responding to thrombin. The co-expression seen in skin is particularly interesting as a possible means of orchestrating the epidermal response to wounding.

A lack of detectable local tissue factor mRNA by *in situ* hybridization does not exclude the presence of sufficient tissue factor protein to locally trigger thrombin generation given the potency of tissue factor and the remarkable amplification that occurs in the coagulation cascade.[11] The redundancy that exists in the triggering mechanisms for the coagulation cascade makes this same point.[12] Moreover,

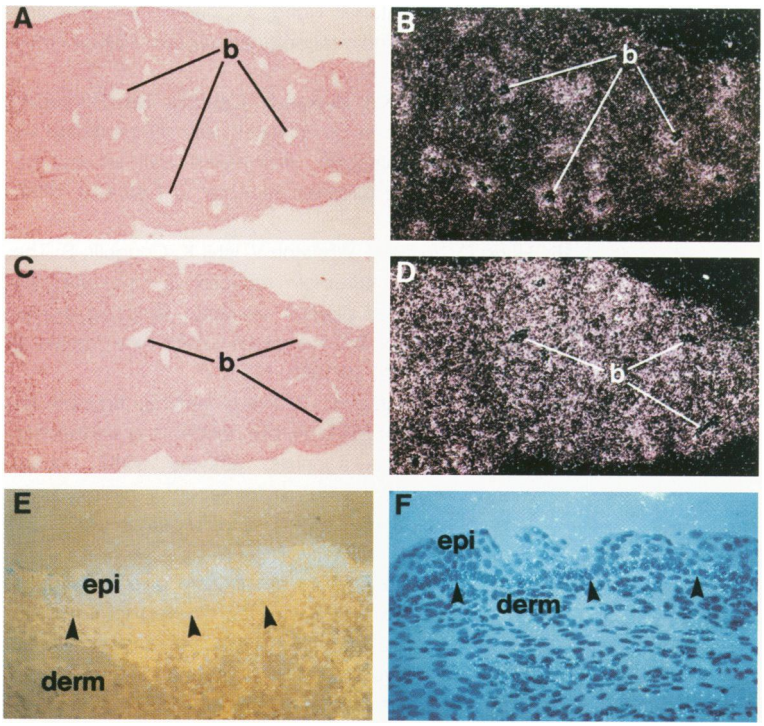

Figure 6. *Co-expression of tissue factor and thrombin receptor in embryonic lung and skin. Embryo sections at 14.5* (**A-D**) *and 16.5* (**E, F**) *days p.c. were hybridized with antisense probes for tissue factor* (**A, B, E**) *or thrombin receptor* (**C, D, F**). *Paired bright and dark-field views of lung* (**A** *and* **C** *versus* **B** *and* **D**, *respectively*) *are shown (magnification ×200). Skin sections* (**E** *and* **F**) *were photographed at ×400 using epi-illumination to visualize the silver grains and transmitted light to visualize the tissue. b, bronchus; epi, epidermis; derm, dermis. Arrows indicate basal cell layer hybridizing for thrombin receptor mRNA.*

the existence of other proteases or even peptide ligands that serve to activate the thrombin receptor cannot be excluded.

Prothrombin Expression in the Embryonic Liver

Prothrombin mRNA expression was limited to the embryonic liver from E12.5 to E16.5 (Figure 1, E-F); no specific hybridization was seen at days 9.5 and 10.5. This observation by *in situ* hybridization in the mouse is consistent with previous Northern and RNAse protection analyses of prothrombin expression during rat development which revealed low to absent prothrombin expression before E18.[26] Because maternal coagulation factors do not freely pass the placenta,[27,28] the absence of detectable prothrombin mRNA before E12.5 despite the broad distribution of thrombin receptor mRNA by E9.5 is noteworthy. It raises the question of whether prothrombin is available to activate embryonic thrombin receptors before the onset of fetal hepatic prothrombin expression and suggests the possibility that other distinct protease or peptide agonists for the thrombin receptor may exist.

After day 12.5, prothrombin and thrombin receptor mRNAs were both expressed in the liver. Prothrombin mRNA was widely expressed in hepatocytes, while thrombin receptor was localized to focal collections of cells and was most highly expressed in very large cells, presumably representing megakaryocytes in hematopoietic islands. Whether local prothrombin conversion and receptor activation occurs is unknown.

Conclusion

These data demonstrate the embryonic localization of mRNAs encoding three key proteins in the thrombin/thrombin receptor system. This descriptive information suggests a number of hypotheses regarding the thrombin receptor's role *in vivo*, hypotheses which remain to be tested. The abundance of thrombin receptor mRNA in the embryonic cardiovascular and nervous systems suggests a possible role for the receptor in the early development of these tissues. The presence of thrombin receptor mRNA in the endocardial cushions and the co-localization of both thrombin receptor and tissue factor mRNA in the embryonic lung and skin suggests that thrombin receptor activation may play a role in local interactions required for the development or repair of these tissues. Localization of receptor mRNA to the endocardial cushions is particularly interesting given thrombin's known mitogenic activity and the frequency of developmental abnormalities involving these structures. Most intriguing of all, the finding of abundant thrombin receptor mRNA expression before detectable

prothrombin expression will stimulate a search for other proteases or peptides capable of acting at this receptor. Last, this study will be extremely helpful for defining and interpreting the phenotype of thrombin receptor knockout mice, which will in turn reveal the role played by the thrombin/thrombin receptor system *in vivo*.

Acknowledgments

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