Progressive and Transient Expression of Tissue Plasminogen Activator During Fetal Development

Eugene G. Levin, Carole L. Banka, Graham C.N. Parry

Abstract—In previous studies of the role of tissue plasminogen activator (tPA) in the lung inflammatory response, we observed that tPA expression was present exclusively in the small arteries and arterioles within the lung and absent from the capillaries, veins, and large pulmonary arteries. To define more completely the expression pattern of tPA, we evaluated the distribution of this protein during prenatal and postnatal development. tPA was first observed in the rat fetus at day 13 in the large arteries of both the thoracic and cranial cavities, including the dorsal aortas and pulmonary arteries in the former and the internal carotid and middle cerebral arteries in the latter. By day 15, tPA was no longer detectable in the aortas but appeared throughout the pulmonary, subclavian, vertebral, and basilar arteries. At day 17, tPA had disappeared from the subclavian artery and the proximal portion of the vertebral artery but was found in the smaller arterial branches of these 2 large vessels. By the end of gestation, tPA had also disappeared from the main pulmonary arteries but remained in the branches at the hilus of the lung. At birth, tPA was concentrated in the endothelia of arteries within the pia mater, the basilar and superficial cerebral arteries, and the lung arterial system. As the animals reached maturity, tPA disappeared from the larger cerebral arteries and their cortical branches but continued to be expressed in the vessels of the pia mater and lung. This study indicates that tPA expression is a dynamic process that responds to a changing arterial environment during vascular development. (Arterioscler Thromb Vasc Biol. 2000;20:1668-1674.)

Key Words: tissue plasminogen activator ■ arteries ■ rat fetus ■ endothelial cells

Numerous studies have established that endothelial cells play a pivotal role in the physiology and pathology of the vascular system.1,2 These cells express a variety of proteins that maintain the structural integrity of the vessel wall, inhibit or promote initiation of the coagulation cascade, and mediate the inflammatory response, as well as other dynamic changes in the vasculature during pathological events. The expression of these proteins in some cases is limited to specific vascular beds or tissues. For example, endothelial cells forming the blood-brain barrier demonstrate complex, tight junctions and specialized transport proteins3,4; in lymphoid tissues, the high endothelium consists of cells that express unique adhesion molecules for the attachment of lymphocytes,5 whereas the endothelia of postcapillary venules mediate adhesive interactions between leukocytes and the vessel wall.6 Recent studies have suggested that tissue plasminogen activator (tPA) is another endothelial cell protein expressed in a specific location within the vascular system. Since its discovery, tPA has been considered the primary activator of the blood fibrinolytic system.7 Plasma tPA has always been thought to be a product of the endothelial cells of all vessels, a conclusion resulting from studies with cultured endothelial cells derived from a variety of tissues and organs.8,9 The validity of this premise, however, is brought into question by recent studies that indicate that tPA is not associated with all endothelia in vivo. Examination of the mouse lung during the inflammatory response clearly showed that both tPA mRNA and protein are absent from endothelial cells of the capillary and venous beds within the lung.10 Even under hyperoxic conditions in which the number of vessels expressing endothelial cell tPA increased, tPA was associated only with the arteries. In addition, the large pulmonary artery was also devoid of tPA, indicating that cells that have been used historically for the study of tPA regulation in vitro may not be producers of this protein in vivo. In contrast to early assumptions about the systemic function of tPA in hemostasis and endothelial cell biology, these results raise the possibility that the role of tPA is more localized and more specific than previously thought.

To determine whether our interpretation of these early studies is correct, ie, that endothelial cell tPA is not the product of endothelial cells in general but provides a more defined role in hemostasis, we assessed the expression pattern of the protein throughout the course of fetal development and after birth. The results demonstrate that tPA is a product of a select subset of arterial endothelial cells. However, during fetal development, expression starts with the aorta and spreads to and subsequently disappears from a variety of

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From the Departments of Molecular and Experimental Medicine (E.G.L.) and Immunology (C.L.B., G.C.N.P.), The Scripps Research Institute, La Jolla, Calif.
Correspondence to Eugene G. Levin, PhD, Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550 N Torrey Pines Rd, La Jolla, CA 92037. E-mail glevin@scripps.edu
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large vessels in the thoracic and cranial cavities. At no time is tPA found in other organs. The final physiological pattern of expression in the adult is confined to the lung and small vessels in the brain, a pattern that suggests a localization more restricted than ever suggested by in vitro studies.

Methods

Materials

The tPA antibody used in this study for immunohistochemical analysis of rat tissue was a rabbit polyclonal anti-human tPA IgG provided by Dr Howard Soule, Corvas International, San Diego, Calif. This antibody at 50 μg/mL was capable of neutralizing at least 1 U of tPA activity for 3 hours in fibrin overlay gels but had no effect on urokinase activity. Rabbit polyclonal antibodies to human von Willebrand factor (vWF) were provided by Dr Zaverrio Ruggeri, The Scripps Research Institute, La Jolla, Calif. Monoclonal antibodies against smooth muscle cell actin and detecting antibodies (sheep anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase) were purchased from Sigma Chemical Co. RNA probes to rat tPA were generated from a 400-bp fragment of rat tPA in pGEM provided by Tor Ny, Umea University, Umea, Sweden.

Animal Experimentation

All animal protocols were approved by the Animal Research Committee (protocol No. ARC480CT8). Timed pregnant rats (Wistar) were placed in a chamber containing halothane (Halocarbon Inc) until respiration ceased, the abdominal cavity was exposed, and the uterus was removed. The fetuses were separated and immediately frozen in LN2. Ovaries were obtained from 5-week-old rats and immediately frozen in LN2.12

Immunohistochemical Staining

For immunohistochemical analysis, tissue sections were dewaxed in xylene, rehydrated, and incubated with 20% nonimmune goat serum (Sigma Chemical Co) for 30 minutes at 37°C. The serum was removed and the tissues incubated with the appropriate dilutions of anti-tPA, anti-αWF, or anti-actin antibody overnight at 4°C. After being washed with PBS, biotinylated sheep anti-rabbit IgG or goat anti-mouse IgG (1:50, Sigma Chemical Co) was added for 30 minutes at 37°C, and the slides were washed and then treated with 3% H2O2 for 20 minutes at room temperature. Antigen-antibody complexes were detected by incubation with Extr-Avidin-peroxidase (1:600, Sigma Chemical Co) for 30 minutes at 37°C, followed by addition of 3-amino-9-ethylcarbazole for 10 minutes at 37°C. All slides were counterstained with hematoxylin. The antigen was visualized as a red precipitate. Immunostaining of a sequentially cut section with nonimmune rabbit IgG was performed in parallel as a negative control in each experiment. Preadsorption of the antibody with purified recombinant tPA (Activase, Genentech) eliminated staining of the tissue, validating the specificity of binding to tPA. Photographs were taken with a Nikon 6000 camera on a Zeiss Axiolab microscope at 50 to 400 magnification.

Results

Developing embryos starting at day 12 were fixed, serially sectioned, and immunostained for tPA to determine the expression pattern during development. At day 12, no tPA antigen was detected in any of the blood vessels despite the fact that the endothelia of all arteries examined had produced and stored vWF (data not shown). Within the next 24 hours, during day 13, the endothelia of various vessels in the thoracic and cranial cavities begin to produce tPA (Figure 1). The endothelia of the dorsal aorta were positive for tPA, although the corresponding cells of the cardinal vein, within the same plane and in the same surrounding environment, were not (Figure 1C). tPA expression was continuous from the aortas into the subclavian (data not shown) and vertebral (Figures 1A and 1D; va) arteries. Moving cranially, we also observed tPA expression in the entire length of the internal carotid artery (which forms the rostral extension of the dorsal aorta at this stage) and the middle cerebral arteries (Figures 1B and 1D). In each case, smooth muscle cell migration into the surrounding tissue had occurred, although the vessel wall remained immature, containing only a single layer. Figure 1C demonstrates early tPA expression within the endothelial cells of each pulmonary artery. tPA expression was absent from the arteries of various other organs, including the heart, liver, and kidneys (data not shown).

By day 15 (Figures 2 and 3), endothelial cell tPA expression disappeared from the aorta but continued to be observed in the arterial endothelia of the pulmonary, cranial, cervical, and thoracic arteries as they developed and elongated. Despite its absence from the aorta, tPA appeared at the junction of the aorta and subclavian artery (Figure 2A; s) and continued to be detectable along the entire length of the vertebral artery (Figure 2A; v). The main pulmonary arteries also remained positive for tPA antigen (Figure 2C; p) as well as the endothelia of arteries developing within the lung parenchyma (Figure 2C; pi). In contrast, the common carotid arteries (Figure 2B; cc), another major branch of the aorta within close proximity to the subclavian, were negative for tPA expression. tPA was now absent in the internal carotid artery after its divergence from the common carotid. This feature is demonstrated in Figure 3D, which shows the lower extremity of the internal carotid artery (ic) within the same plane as the cochlear canals. However, as the internal carotid artery extends caudally and decreases in diameter, tPA appears in its endothelium and continues to be expressed into the middle cerebral artery (Figure 3E; ic and mc). At this level, the internal carotid artery is juxtaposed to the base of the brain (notice the pituitary, p). Thus, tPA-positive and tPA-negative cells are found within the same artery (represented by sections d and e in the diagram of the fetus). Also shown in Figure 3D is a section of the medulla, demonstrating that the endothelia of the vertebral arteries (v), the basilar artery (b), and the cerebellar arteries (c) are all positive for tPA. tPA expression was not confined to these larger arteries; the arterioles (Figure 3F; a) formed from the middle cerebral artery and surrounding the ventricular system express tPA. This finding is in contrast to the small veins that are present within the same plane and are of similar size (v).

By day 17, both the vertebral and subclavian arteries within the thoracic cavity became devoid of tPA (Figures 4A and 4C). However, tPA expression appeared in a branch of the vertebral artery, a pattern (ie, branch-point stimulation) that we have found to occur in different vascular beds during development and in the mature animal.10 Despite the absence of tPA from the vertebral arteries at the proximal region, the protein reappeared as the vertebral arteries extended caudally toward the brain (Figure 4D; v), in a similar fashion to that
observed with the internal carotid artery at day 15 (Figure 3F). Thus, tPA expression is not simply a function of the type of vessel in which the endothelial cells are found but may respond to factors that change throughout the length of a single artery.

After day 17, we did not observe any major changes in the pattern of tPA expression save one. By day 20, tPA disappeared from the endothelia of the large pulmonary arteries (Figure 4B; pa) but continued to be expressed as they branched into the lung parenchyma, another example of

**Figure 1.** Embryonic day 13. A, Section through the thoracic cavity: dorsal aorta (d), vertebral artery (va), cardinal vein (cv), trachea (t), esophagus (e), neural tube (nt), and pulmonary arteries (p). B, Section through the cranium showing the internal carotid (ic) and middle cerebral (mc) arteries and convergence of the 2 (*). 4v indicates fourth ventricle; v, vein; mv, mesencephalic vesicle; and de, diencephalon. C, tPA staining is found in the dorsal aorta (d) but not in the cardinal vein (cv). The pulmonary arteries (p) have tPA-positive endothelial cells. D, Endothelium of the middle cerebral/internal carotid artery (corresponding to asterisk in Figure 1B) are positive for tPA. The vertebral artery shown in A was stained for tPA. Magnification: A and B, ×50; C and D, ×400.

**Figure 2.** Embryonic day 15. A, Section through the upper region of the thoracic cavity showing branch points of the aorta, subclavian (s), and vertebral (v) arteries. Endothelial cells of the aorta are negative for tPA, but tPA-positive cells appear as the subclavian artery forms and then branches into the vertebral artery. vc indicates vena cava; e, esophagus. B, Section showing that the common carotid arteries (cc) are negative for tPA. t indicates trachea; e, esophagus. C, Section through the thoracic cavity showing tPA in the endothelium of the pulmonary arteries (p) both external to and within (p) the developing lung. a indicates aorta; vc, vena cava; t, trachea; and e, esophagus. Magnification ×200.
branch-point stimulation. At this time, the pulmonary arteries that align with the bronchi and bronchioles were all expressing tPA. This result establishes the distribution of tPA within the pulmonary system that exists into adulthood.10

After birth, tPA is exclusively associated with the arteries of the pulmonary system and the central nervous system. Within the central nervous system, tPA is found in the distal portions of the major arteries furnishing blood to the brain, including the internal carotid, vertebral, and basilar arteries as well as other major branches, such as the anterior and middle cerebral and cerebellar arteries (not shown). In addition to these larger arteries, the arterioles of the pia mater, shown at the bottom surface of the brain section (Figure 5A, filled arrows), also express tPA. In fact, we have found that the pial vasculature is highly enriched in tPA throughout the brain, and branches of the pial vessels continue to express tPA as they infiltrate into the brain parenchyma. Although the pial vessels are a rich source of tPA, the number of vessels within

Figure 3. Embryonic day 15. D, Within the plane of the cochlear canals (cc), the internal carotid arteries (ic) are negative for tPA, but the vertebral arteries (v), basilar artery (b), and cerebellar arteries (c) within the medulla are positive. sc indicates spinal cord. Insert, Higher magnification of the left internal carotid artery. E, Section showing that the internal carotid (ic) and middle cerebral (mc) arteries are positive for tPA at the base of the brain. p indicates pituitary; 3v, third ventricle; and tg, trigeminal ganglion. F, Brain tissue showing small arteries (a) and veins (v), the former being positive for tPA and the latter, negative. The arteries are identified as vessels of the pia mater. Magnification ×200.

Figure 4. Embryonic days 17 and 20. A, Section through the thoracic cavity showing that the subclavian artery is now negative for tPA. s indicates subclavian; vc, vena cava; th, thymus; e, esophagus; t, trachea; and in, innominate artery. B, The main pulmonary arteries (pa) are negative but become positive as they branch at the hilus of the lung. t indicates trachea. C, At this level, the vertebral artery (v) is no longer positive for tPA. However, tPA appears as a smaller vessel branches from the vertebral artery. sc indicates spinal cord. D, The vertebral artery (v) shows positive staining for tPA at this region closer to the brain. The internal carotid artery (ic) is negative. Magnification ×100.
the brain parenchyma that contain tPA is minimal. The number of vessels expressing vWF in the medulla (Figure 5B) was compared with those expressing tPA (Figure 5A) within serial sections. It is clear that the number of vessels containing tPA (open arrows) are few compared with the total number. In sections cut from the region containing the hippocampus (Figures 5C and 5D), none of the vessels within the parenchyma were positive for tPA (Figure 4D) when compared with the arteries demonstrated by staining with smooth muscle cell actin (Figure 4C). The tPA-positive vessels are associated with the membrane that lines the interface between the tissue layers. At this stage in development, tPA expression is largely relegated to the arteries of the pial membrane and large vessels.

As the animals reach adulthood, there is a shift in the types of vessels in the brain that express tPA. At 4 months of age, tPA continues to be observed in vessels of the meninges and appears in a small fraction of vessels within the brain parenchyma (shown in Reference 13). In contrast to the pial vessels, the vertebral and basilar arteries as well as the larger cortical branches of the major cerebral arteries become negative (data not shown). Thus, as the animal ages, there appears to be a shift away from tPA expression in the larger arteries supplying blood to the different parts of the organ to the smaller, more localized vessels.

**Discussion**

Our analysis of endothelial cell tPA expression in the whole animal demonstrates that this is a protein with very limited distribution within the vascular system, being associated with a fraction of the vessels at any 1 time during prenatal and postnatal life. During prenatal development, tPA expression is not static but appears in a progressive and transitory pattern that is restricted to the arterial endothelial cells of the thoracic and cranial cavities (a summary of the data in graphic form is shown in Figure 6). tPA first appeared on the 13th day in the endothelia of the dorsal aortas, the subclavian arteries, and the internal carotid arteries (which at this time form the rostral extremity of the aorta). Within 24 hours, the aortic endothelia were no longer positive, but expression had spread to the larger arteries supplying the brain (vertebral, basilar, and middle cerebral) as well as the main pulmonary arteries. By the end of gestation, tPA expression had disappeared from all of the vessels of the thoracic cavity, except the arteries within the lung, but remained in vessels closely associated with tissues of the central nervous system: the vertebral, internal carotid, middle cerebral, and spinal arteries and the arterioles of the pia mater. Within a few weeks of birth, the endothelia of all of the larger vessels, including the vertebral and basilar arteries and the larger cerebral vessels, were no longer expressing tPA. In addition to establishing that tPA expression is narrowly distributed within the vascular system, the data also suggest that the function of tPA may be far more limited than previously assumed. Since its discovery, tPA has been considered to be the primary activator of the blood fibrinolytic system. The dissolution of thrombi was a function of the tPA-dependent conversion of plasminogen to plasmin that culminated in the degradation of fibrin and other proteins incorporated into the clot. This conclusion was based primarily on in vitro studies and animal studies of the use of tPA in therapeutic thrombolysis, where tPA was infused into the animal and its efficacy for dissolving preformed thrombi was assessed, however, by results from tPA-deficient mice, which showed that thrombus dissolution was not dramatically affected by the absence of tPA. Although the rate of thrombolysis was reduced, clot resolution did occur. This latter result has been explained by the concept of redundancy, in which other proteins, such as the plasminogen activator urokinase, with similar or identical functions, replace the function of the targeted protein. However, with the results presented here, we propose that this may not be the case, and that localized proteolysis, and not systemic vascular fibrinolysis, is the primary function of tPA. One question that remains, however, is whether the absence of tPA in the endothelium is due to a true lack of expression or whether tPA antigen is below the level of detection. Previously
published reports describing selective tPA expression in the arterioles of the adult lung suggest that the former possibility is likely the case.\textsuperscript{10} In those studies, lung sections were analyzed immunohistochemically, by in situ hybridization, and by fibrin overlay activity measurements. In all 3 cases, tPA antigen, mRNA, and activity were found only in the endothelium of the pulmonary arteriole system and colocalized in all cases. Fibrin overlay assays have the advantage of allowing long-term incubation of the sections (days) and are highly sensitive to small amounts of plasminogen activator. These data, although not conclusively proving the absence of tPA, strongly support our proposal that tPA is expressed only in the endothelial cells displaying positive antibody staining.

We also suggest that the reproducible pattern of expression in the lung and pia mater represents constitutive expression of tPA. However, we have shown that stimulation of tPA expression in vivo can also occur, eg, during hyperoxia, which induces an increase in tPA expression in other arterioles within the lung parenchyma.\textsuperscript{10} Thus, even when tPA expression can be stimulated, it is found only in the vessels that it is associated with under normal conditions.

The rapid changes in tPA expression within the same vessels during vascular development suggest that the control of tPA gene expression is influenced by changing environments as the fetus matures, rather than sublineage (clonality) of the endothelial cells,\textsuperscript{17,18} although the regulatory network controlling localized tPA production might be lineage-specific. The environments must themselves be highly localized and selective for small areas within the vascular system. In some cases, the apparent differences in environment are easily discernible. For example, the branch points between the vertebral artery and its branches and the bifurcation of the main pulmonary artery at the hilus of the lung are areas in which sudden and dramatic changes occur in vessel size and luminal hemodynamics. Changes in vessel wall shear and pressure are known to promote the gene transcription of tPA in endothelial cells via defined regulatory elements.\textsuperscript{19,20} On the other hand, the appearance of tPA in the distal but not proximal extremities of the vertebral artery does not have an obvious explanation. No branching, bifurcation of the vessel, or sudden change in orientation occurs, although luminal diameter does narrow gradually as the vessels approach the cranium. Our previous work suggests that vessel size may be a factor in tPA expression.\textsuperscript{13} In a study of the relationship between tPA and the vasculature of the brain, it was found that although very few vessels express tPA (<3%), 90% of those were in vessels between 7 and 30 μm in diameter. Thus, specific vessel diameter or some consequence of that parameter may be a criterion for tPA expression. Another possibility that is being examined is the
effect of the brain on these endothelial cells and their expression of tPA. Such a suggestion is based on other examples of endothelial cell gene expression regulated by cell-cell interaction. For example, formation of the blood-brain barrier appears to be a function of the interaction of endothelial cells and astrocytes,21 while the same appears to be true with vWF expression and endothelial cell–myocyte interaction.22,23 Thus, the control of endothelial cell gene expression by environmental factors found within a well-defined vascular bed has been identified.

The problem with attempting to predict which of these factors is responsible for the onset of tPA production is that there are no consistently shared characteristics among vessels of similar types. During development, for example, vessels of similar dimensions and hemostatic conditions as those positive for tPA exist in other organs, but no tPA has been found in these other tissues. The expression of tPA in endothelial cells of the pulmonary and systemic circulation also reduces the possibility of mechanical (ie, pressure) effects as a reason, since the hemostatic properties of these 2 circulatory systems are different. Thus, general physical characteristics of the vessel and its lumen cannot be linked to the control of tPA expression. Therefore, serious consideration must be given to the possibility that brain- and lung-specific factors play an important role in tPA expression. In the end, it is conceivable that a compendium of several factors regulates tPA expression in the endothelial cells of these vessels.

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