American Journal of Pathology, Vol. 149, No. 1, July 1996

Tissue Factor Expression during Human and Mouse Development

Thomas Luther,* Conrad Flössel,* Nigel Mackman,† Angelika Bierhaus,* Michael Kasper,* Sybille Albrecht,* E. Helene Sage,‡ Luisa Iruela-Arispe,§ Heinz Grossmann,* Arne Ströhlein,* Youming Zhang,‖ Peter P. Nawroth,‖ Peter Carmeliet,¶ David J. Loskutoff,† and Martin Müller*

From the Institute of Pathology, Technical University Dresden, Dresden, Germany, the Departments of Immunology and Vascular Biology,† Scripps Research Institute, La Jolla, California, the Department of Biological Structure,‡ University of Washington, Seattle, Washington, the Department of Pathology,§ Beth Israel Hospital, Boston, Massachusetts, the Department of Medicine I,‖ University Heidelberg, Heidelberg, Germany, and the Center for Transgene Technology and Gene Therapy,¶ Leuven, Belgium*

In the adult organism the cellular distribution of tissue factor (TF) expression corresponds to biological boundary layers forming a hemostatic barrier ready to activate blood coagulation after tissue injury. Whether TF expression might also play a role in development is unknown. To determine the significance of TF in ontogenesis, we examined the pattern of TF expression in mouse development and compared it with the distribution of TF in human post-implantation embryos and fetuses of corresponding gestational age. At early embryonic periods of murine (6.5 and 7.5 pc) and human (stage 5) development, there was strong expression of TF in both ectodermal and entodermal cells. **In situ** ***hybridization and immunohistochemistry demonstrated that TF mRNA and protein were expressed widely in epithelial areas with high levels of morphogenic activity during early organogenesis. Staining for TF was seen during ontogenetic development in tissues such as epidermis, myocardium, bronchial epithelium, and hepatocytes, which express TF in the adult organism. Surprisingly, during renal development and in adults, expression of TF differed between humans and mice. In humans, maturing stage glomeruli were stained for TF, whereas in mice, TF was absent from glomeruli but was present in the epithelia of tubular segments. In neuroepithelial cells, there was a substantial expression of TF. Moreover, there was robust TF expression in tissues such as skeletal muscle and pancreas, which do not express it in the adult. In contrast, expression of the physiological ligand for TF, factor VII, was not detectable during early stages of human embryogenesis using immunohistochemistry. The temporal and spatial pattern of TF expression during murine and human development supports the contention that TF serves as an important morphogenic factor during embryogenesis. (Am J Pathol 1996, 149:101–113)***

Tissue factor (TF) is the primary cellular initiator of blood coagulation.[1] This 47-kd glycoprotein is the receptor and essential cofactor for coagulation factor VII/VIIa, a soluble serine protease (reviewed in Refs. 1 and 2). Although there is a structural homology with some cytokine receptors, TF appears to be unrelated to any other known procoagulant and anticoagulant proteins, suggesting an evolutionary origin for TF separate from that of other coagulation factors.[2] TF is expressed by many cell types that are not exposed directly to flowing blood and is particularly abundant in the parenchyma of brain, lung, and placenta.[3,4] Immunohistochemistry and *in situ* hybridization demonstrated that, in the adult organism, TF is expressed in the extravascular cells of many tissues[5–11] such as epidermis, astroglia, bron-

Part of this work was presented at the 39th Annual Meeting of the GTH, Berlin, Germany, and published as an abstract in Annals of Hematology 1995, 70: A76.

Supported by a grant from the Sächsisches Staatsministerium (T. Luther; A. Bierhaus). P. P. Nawroth was supported by a Heisenberg Stipend (DFG). D. J. Loskutoff was supported by HL 47819 and N. Mackman was supported by HL16411.

Accepted for publication February 26, 1996.

Address reprint requests to Dr. Thomas Luther, Institute of Pathology, Technical University Dresden, Fetscherstr. 74, D01307 Dresden, Germany.

chial epithelia, mucosal epithelial layers, and adventitia of vessels. The cellular distribution of TF expression corresponds to biological boundary layers that may form a hemostatic barrier ready to activate blood coagulation after tissue injury.[5]

From a teleological point of view, this distribution pattern of TF might be explained as part of a protective system in terms of hemostasis.[1,12] However, the TF gene has been classified as an immediate-early gene responsive to serum and cytokines.[13,14] Various cell types, including monocytes and endothelia, that do not express TF under normal physiological conditions can be induced to express TF by stimuli such as endotoxin, cytokines, and immune complexes.[15-22] These findings indicate a link between inducible TF expression in those cells and inflammatory tissue reactions. In recent studies it was shown that expression of TF in mouse and human tissues known to express TF under normal physiological conditions is also regulated positively by cytokines, growth factors, and hormones.[23-25] Moreover, the nonuniform distribution of TF throughout the body indicates that TF expression is a function of the differentiation in various cell types.[5,6,10,26]

TF appears to be the only protein in the coagulation pathway for which a congenital deficiency has not been reported.[2,27] This leads to the postulate that TF might have additional properties that are essential to normal embryonic development; therefore, its absence may not be compatible with survival.[13,14,28] To date, little is known about the role of cellular TF expression in embryogenesis because few studies have examined TF distribution in mammalian embryos.[29,30] As developmental expression of TF may provide new insights into its functions, we studied the localization of TF mRNA and protein during murine development and compared it with the TF protein distribution in human embryos of corresponding stages.

Materials and Methods

Tissue Samples

Balb/c and C57BL/6 mouse embryos (6.5, 7.5, 8.5, 9.5, 10.5, 11.5, 12.5, 13.5, 14.5, 15.5, and 18.5 days post-conception (pc); n = 40) were fixed in 4% neutral buffered formaldehyde for 3 hours or overnight and were embedded in paraffin.

Human adult, juvenile, embryonic, and fetal tissues were obtained at surgery and abortions according to regulations of the Ethics Committee of the Medical Faculty. The tissues were fixed in 4% neutral buffered formalin and were embedded routinely in paraffin. The developmental and gestational age of the examined embryos and fetuses was determined by morphological staging according to Carnegie stages and to the crown-rump length and according to clinical statement of the first day of the last menstrual period before conception (15, 30, 50, and 56 days after ovulation and 9, 10, 11, 12, 16, 20, 21, 24, and 28 weeks of development; n = 13).

Antibodies

A polyclonal anti-mouse TF serum was generated in rabbits using recombinant mouse TF (amino acids 1 to 294).[31] The purified immunoglobulin fraction of the antiserum recognizes a single band of approximately 43 kd in both immunopurified murine lung TF apoprotein and crude extract from murine skin (Western blot analysis; Figure 1A). The specificity of the antibody in immunohistochemistry was confirmed by competitive inhibition of the immunohistological reaction using immunopurified murine lung TF apoprotein (Figure 1, B and C).

Human TF-specific monoclonal antibodies[32] (American Diagnostica, Greenwich, CT) were used for immunohistochemistry on paraffin-embedded material as culture supernatants, as recently described.[10] A human factor-VII-specific monoclonal antibody (VC11) was raised against purified factor VII as described previously.[33] The monoclonal antibody recognizes a single band of approximately 55 kd on immunoblots (S. Albrecht, unpublished observation) with both purified factor VII (Novo Nordisk, Bagsvaerd, Denmark) and human plasma.

For detection of cytokeratin (CK), we used the monoclonal antibody MNF 116 (Dakopatts, Hamburg, Germany; preferably reacting with CK8).

Western Blot Analysis

Immunopurified murine lung TF and homogenized murine skin were boiled in Laemmli sodium dodecyl sulfate (SDS) reducing buffer. After centrifugation for 10 minutes at 30,000 × *g*, the supernatant proteins were run on 12% Tris-glycine-SDS-polyacrylamide gels (Novex, San Diego, CA) and were blotted onto a nitrocellulose membrane (BA85, Schleicher & Schuell, Dassel, Germany). After a blocking step with undiluted fetal calf serum, the proteins were detected with the rabbit anti-mouse TF immunoglobulin (diluted 1:100, 25 μg/ml) and peroxidase-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany; diluted 1:5000). Peroxidase activity was visualized with diaminobenzidine and nickel enhancement (SERVA, Heidelberg, Germany).

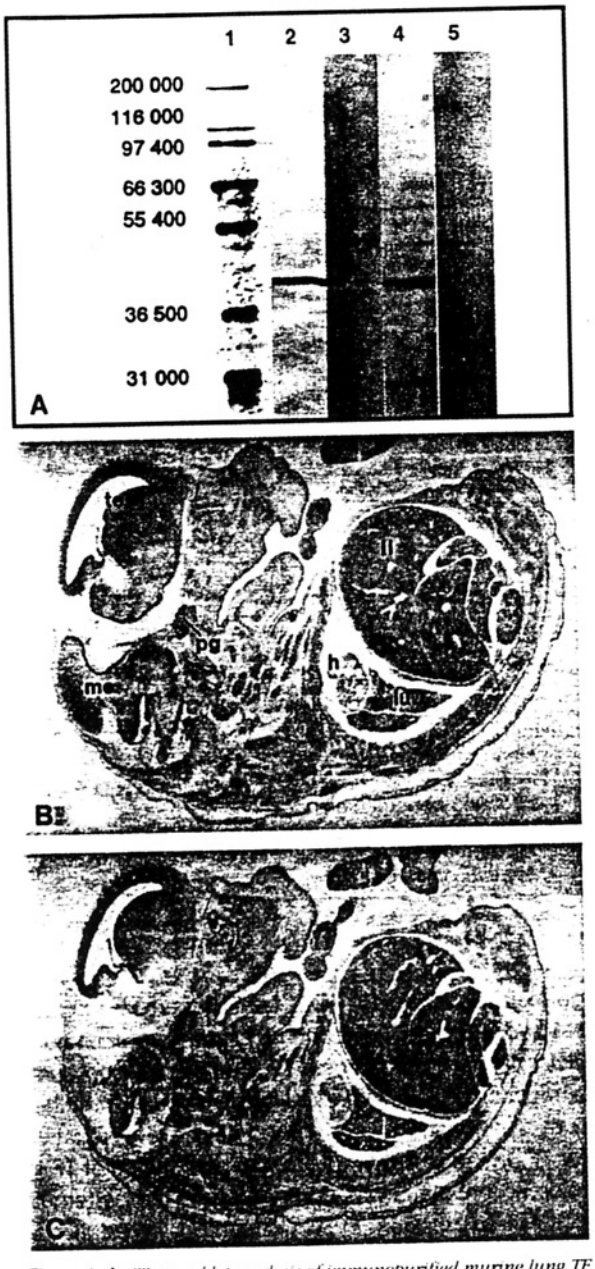


Figure 1. A: *Western blot analysis of immunopurified murine lung TF* (lanes 2 *and* 3) *and crude extract from murine skin* (lanes 4 *and* 5). *The blots were incubated with a polyclonal anti-mouse TF immunoglobulin* (lanes 2 *and* 4) *or a rabbit control immunoglobulin* (lanes 3 *and* 5). B *and* C: *Immunohistochemical staining of TF in sagittal sections of a mouse embryo at 13.5 days pc with anti-mouse TF immunoglobulin as described in Materials and Methods. Positive signal appears as a brown immunoperoxidase reaction product. Competitive inhibition* (C) *of TF-specific staining with immunopurified murine lung TF apoprotein; magnification,* ×15 (B *and* C). *li, liver; lu, lung; h, heart; te, telencephalon; me, mesencephalon; pg, pituitary gland anlage.*

Immunohistochemistry

Paraffin sections (4 μm thick) were mounted on slides that were coated with silane. After a drying step overnight at 37°C, the sections were dewaxed and irradiated in a microwave oven in 0.01 mol/L sodium citrate buffer (pH 6.0), twice for 5 minutes each at 1500 W (Micro-Chef oven FM 3910 Q, type 151, Moulinex, Bagnolet, France), as previously described.[34] After washes in phosphate-buffered saline (PBS) for 5 minutes, the sections were treated with 0.3% H_2O_2 for 30 minutes and were incubated with 30% fetal calf serum in PBS for 20 minutes.

For detection of murine TF, the slides were incubated with a polyclonal anti-mouse TF immunoglobulin[31,35] for 16 hours at 4°C. Sections were washed twice, and peroxidase-labeled goat anti-rabbit IgG (HRP 77; H. Grossmann, diluted 1:400) was used as the secondary reagent. The peroxidase activity was visualized with diaminobenzidine. Negative controls included omission of the primary antibody or replacement by nonimmune rabbit IgG (data not shown).

For detection of human TF, human factor VII, and CK8, the immunoperoxidase procedure was performed as described previously[10,34] with a Vectastain Elite kit (Vector Laboratories, Burlingame, CA). Briefly, monoclonal anti-human TF antibodies were applied overnight at 4°C. Thereafter, the slides were washed with PBS and incubated in diluted biotinylated horse anti-mouse IgG for 15 minutes at 37°C. After several washes, the Vectastain Elite ABC reagent was applied for 15 minutes at 37°C and the washing steps were repeated. Color was developed with diaminobenzidine (Aldrich Chemical Co., Milwaukee, WI) for 10 minutes at room temperature and counterstaining was performed with hematoxylin.

Riboprobe Generation

The murine TF cDNA probe was an 821-bp fragment isolated from plasmid pcmTF2253.[36] This fragment was subcloned into the vectors pGEM-3Z and pGEM-4Z (Promega Corp., Madison, WI) and utilized as a template for *in vitro* transcription of radiolabeled sense (pGEM-3Z) and antisense (pGEM-4Z) TF riboprobes employing SP6 polymerase (Promega) in the presence of [^{35}S]UTP (>1200 Ci/mmol; Amersham, Arlington Heights, IL). Templates were removed by digestion with RQ1 DNAse (Promega) for 15 minutes at 37°C, and the riboprobes were purified by phenol/chloroform extraction and ethanol precipitation.

In Situ *Hybridization*

In situ hybridization was performed as described elsewhere.[9] Briefly, paraffin sections (2 to 5 μm thick) were mounted on slides that were coated with polylysine. Sections were pretreated sequentially

with xylene (three times for 5 minutes each in 2× standard saline citrate (SSC; 0.3 mol/L NaCl, 30 mmol/L sodium citrate, pH 7.0, containing 10 mmol/L 2-mercaptoethanol, 1 mmol/L EDTA) for 10 minutes, paraformaldehyde (10 minutes at 4°C), and proteinase K (1 μg/ml in 500 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.0) (for 10 minutes). Slides were prehybridized for 2 hours in 100 μl of prehybridization buffer (50% w/v formamide, 0.3 mol/L NaCl, 20 mmol/L Tris-HCl, pH 8.0, 5 mmol/L EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 10% w/v dextan sulfate, 10 mmol/L dithiothreitol) at 42°C. Prehybridization buffer (20 μl, containing 2.5 mg/ml tRNA and 600,000 cpm of the ^{35}S-labeled riboprobe) was added, and the slides were hybridized for 18 hours at 55°C. After hybridization, slides were treated with 2× SSC containing 10 mmol/L 2-mercaptoethanol, 1 mmol/L EDTA (twice for 10 minutes each), RNAse A (20 μg/ml in 0.5 mmol/L NaCl, 10 mmol/L Tris-HCl) for 30 minutes, 2× SSC (10 mmol/L 2-mercaptoethanol, 1 mmol/L EDTA) twice for 10 minutes each, 0.1× SSC (10 mmol/L 2-mercaptoethanol, 1 mmol/L EDTA) for 2 hours at 60°C, and 0.5× SSC (twice for 10 minutes each). Finally, the slides were dehydrated by immersion in a graded alcohol series containing 0.3 mmol/L ammonium acetate, dried, coated with NTB2 emulsion (Kodak, Rochester, NY; 1:2 in water), and exposed in the dark at 4°C for 12 weeks. Slides were developed for 2 minutes in D19 developer (Kodak), fixed, washed in water (three times for 5 minutes each), and counterstained with hematoxylin and eosin. Consecutive sections were analyzed with TF sense probe as a control for nonspecific hybridization. No specific signal could be detected in these control hybridizations (data not shown).

Results

To detect TF mRNA and protein expression during ontogenetic development, we performed immunohistochemistry and *in situ* hybridization on sections of mouse as well as human embryos and fetal tissues. In general, TF was expressed in post-implantation stages of both mouse and human development in all embryos examined. During early development, TF was distributed in differentiated cells of all three germ layers (Table 1) but later became more restricted. Although we found no tissue types that consistently do not express TF during development, some more differentiated cells, ie, vascular endothelium, fibroblasts, and red blood cells, were negative for TF. In the prenatal period, the distribution of TF in mice and humans was comparable to the pattern of cellular expression of TF observed in adult tissues.[5,6,10]

Table 1. *Immunohistochemical Staining of TF in Paraffin-Embedded Murine and Human Embryos*

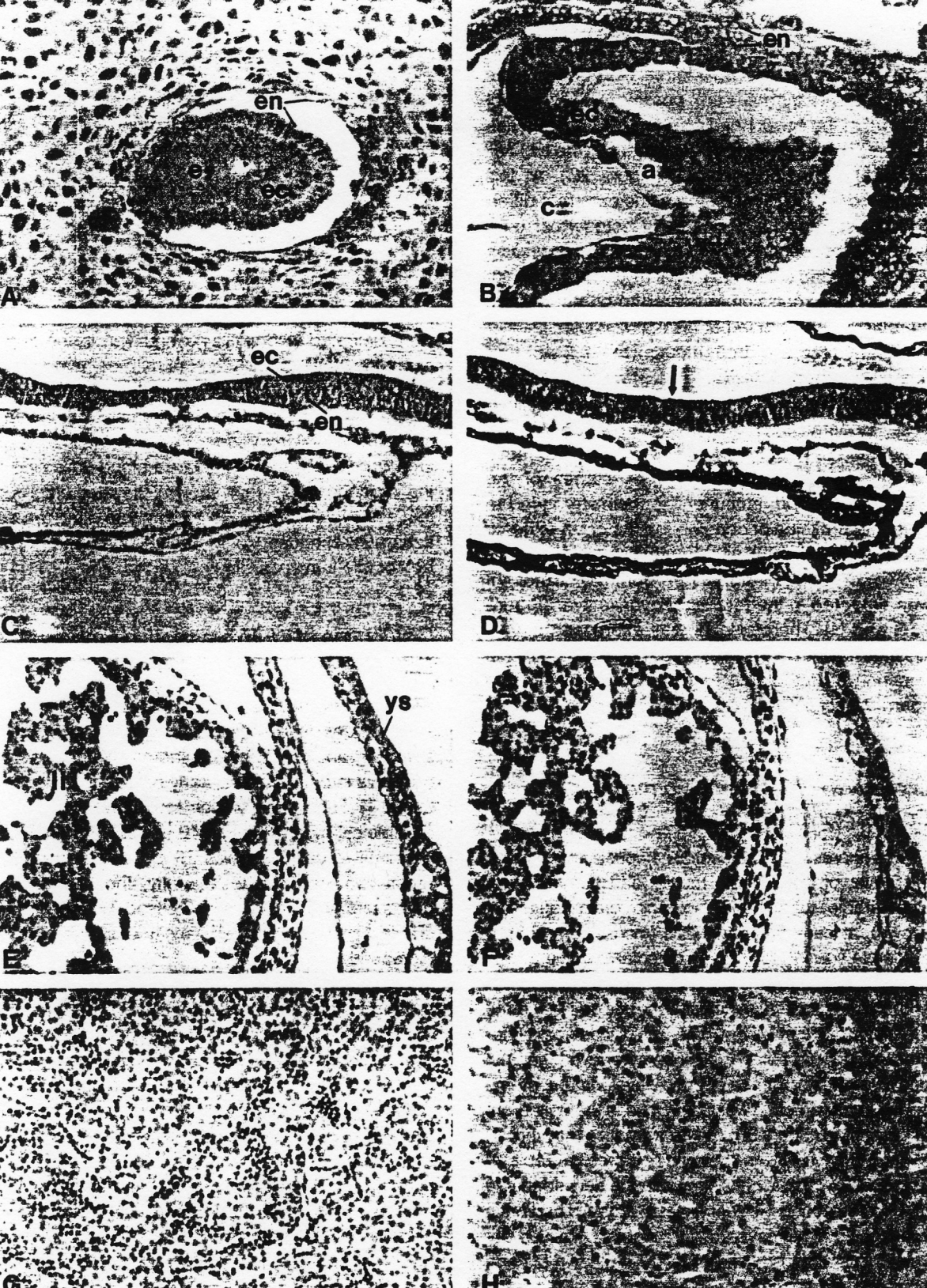
	Embryonic tissues	
	Murine 9.5 to 14.5 days pc	Human stages 12 to 22
Epidermis		
Basal cell layer	+	+
Intermediate layer	++	++
Periderm	++	++
Esophagus		
Epithelia	+	+
Gut		
Epithelia of intestinal tube	++	+
Subepithelial tissue	(+)	−
Smooth muscle anlage	(+)	(+)
Nervous tissues		
Neuroepithelial cells	++	+
Epithelium of choriod plexus	++	++
Leptomeninges	+	+
Glial cells	++	++
Liver		
Hepatocytes	++	++
Lung		
Bronchial tree	+	(+)
Pneumocytes	−	−
Heart		
Heart muscle	+	+
Pancreas		
Exocrine pancreas anlage	++	++
Kidney		
Glomerular epithelia	−	++
Tubular epithelia	++	(+)

Staining reactions were evaluated as follows: −, lack of staining; (+), weak staining; +, moderate staining; ++, strong staining.

TF Expression in Early Post-Implantation Embryos

We could demonstrate robust expression of TF in 6.5- and 7.5-day mouse embryos by immunohistochemistry (Figure 2, A and B). Strong staining of both

Figure 2. *Immunohistochemical detection of TF expression in murine* (A *and* B) *and human* (C *and* E) *early post-implantation embryos (immunoperoxidase procedure).* A *and* B: *Murine embryo sections at 6.5 and 7.5 days pc, respectively. Note the strong staining of both visceral and parietal entoderm (en) as well as the primary ectoderm (ec). e, extraembryonic ectoderm; a, amniotic cavity; c, extraembryonic coelom.* C *and* D: *Sections of a human embryonic disc (stage 5).* C: *TF in entodermal (en) and ectodermal (ec) cells.* D: Arrow *indicates CK8-positive ectodermal cells.* E *and* F: *Sections of a human embryonic liver (stage 12) stained with anti-TF monoclonal antibody* (E) *and anti-factor VII monoclonal antibody* (F). *Liver cells and visceral wall of the yolk sac express TF but no factor VII at this stage of development. li, liver; yc, yolk sac.* G *and* H: *Sections of fetal (11 weeks) and juvenile liver (10 years), respectively, stained with anti-factor VII monoclonal antibody. Magnification ×220* (A), *×380* (B), *and ×190* (C *to* H).


en
e
ec
A
en
ec
a
c
B
ec
en
C
D
ys
E
F
G
H

the parietal and visceral entoderm (distal and proximal wall of the yolk sac, respectively) as well as the primary ectoderm was evident. The mesoderm formed by migration of ectodermal cells between the ecto- and entoderm was also positive for TF (data not shown). In the corresponding stage of human development, TF was already demonstrable in the two cell layers of the embryonic disc (Figure 2C). At this stage of differentiation, there was no apparent difference in staining for TF between ectodermal cells (epiblast) as decorated by cytokeratin expression (Figure 2D) and entodermal cells (hypoblast). In both species, TF expression appeared to be not only on the plasma membrane but also intracellular.

In early stages of both mouse (day 9.5 pc) and human organogenesis (stage 12), we could detect a staining for TF in various tissues including liver cells, visceral layer of the yolk sac (Figure 2E), heart anlage (Figure 3, F and G) and epithelia (Figure 4A). In contrast, at early stages of the embryonic period, immunohistochemistry failed to detect expression of coagulation factor VII either in the liver (Figure 2F) or in other tissues (data not shown) of human embryos. In contrast, factor VII was detected in the fetal and juvenile liver (Figure 2, G and H). Therefore, TF was expressed in various tissues during early organogenesis, in the absence of detectable amounts of its only known ligand factor VII.

Expression of TF during Formation of the Central Nervous System

TF was expressed in the central nervous system during the development of both mouse and human brain. Comparable to human embryonic brain (data not shown), at early organogenesis (mouse, day 8.5), a moderate staining for TF was detectable in the germinal layer of neuroepithelial cells surrounding the lumen of the neural tube (ventricular zone) as well as in the marginal zone formed by processes of these cells (Figure 3A). A more refined resolution, however, was not possible. At later stages of brain development, prominent expression of TF was also found in the epithelium of the human (Figure 3B) as well as mouse (not shown) choriod plexus and in the meningeal cells (Table 1; Figure 3C), which is consistent with the described role of TF in the control of hemostasis at the blood/brain interface.[5,8,10] Moreover, at later stages of mouse development, a diffuse staining for TF protein in the embryonic brain with the highest levels of TF mRNA in the ventricular zone were demonstrable, indicating TF expression in immature glial cells (Figures 3C and 5B). Interestingly, TF was also expressed by neurons in embryonic cranial nerve nuclei and dorsal root ganglia (Figure 3, D and E).

TF Expression in Cardiovascular System during Early Organogenesis

Previous morphological studies have shown that cardiac cells arise from mesodermal precursors in the anterior part of the embryo.[37] We found that TF was detectable early in the developing heart. At early stages of mouse development (days 9.5 and 12.5 pc), TF protein (Figure 3F) and TF mRNA (Figure 5C) were detected with similar expression in the corresponding stage (stage 12) of human embryogenesis (Figure 3G). TF was localized predominantly in myocardial cells in both murine and human heart anlage. In addition, TF expression was demonstrable in smooth muscle cells of the large blood vessels during organogenesis (not shown). In later stages of mouse development (days 13.5 and 18.5 pc), as well as in human fetuses, only weak staining for TF was detectable in myocardium.

Distribution of TF Expression in Embryonic Epithelia

One characteristic of TF expression in adult mice and humans is a strong expression of TF in the stratum granulosum of the epidermis. During embryogenesis in both species, early epidermal cells contained TF, regardless of their assembly into 1-, 2-, or multiple cell layers (Figures 4B and 5D). Whereas the germinative cell layer was TF positive in the early embryonic stages, subsequently, the intermediate cell layers and periderm expressed TF prominently (Table 1). Generally, the intensity of staining for TF increased with maturation.

During the development of the gastrointestinal system, disparate expression of TF was found in the primitive epithelium. At early stages of embryogenesis (mouse, day 11.5 pc; human, stage 12), there was a striking expression of TF protein and mRNA in the epithelia of the stomodeum near Rathke's pouch (Figures 4A and 5A). Later (mouse, day 14.5 pc; human, stage 22), strong staining for TF was found in the adenohypophysis anlage arising from Rathke's pouch (Figure 4, C and D). In addition, prominent expression of TF mRNA and protein in the epithelium was observed during differentiation of the caudal components of the foregut, which include the esophagus, respiratory system, stomach, duodenum, pancreas,

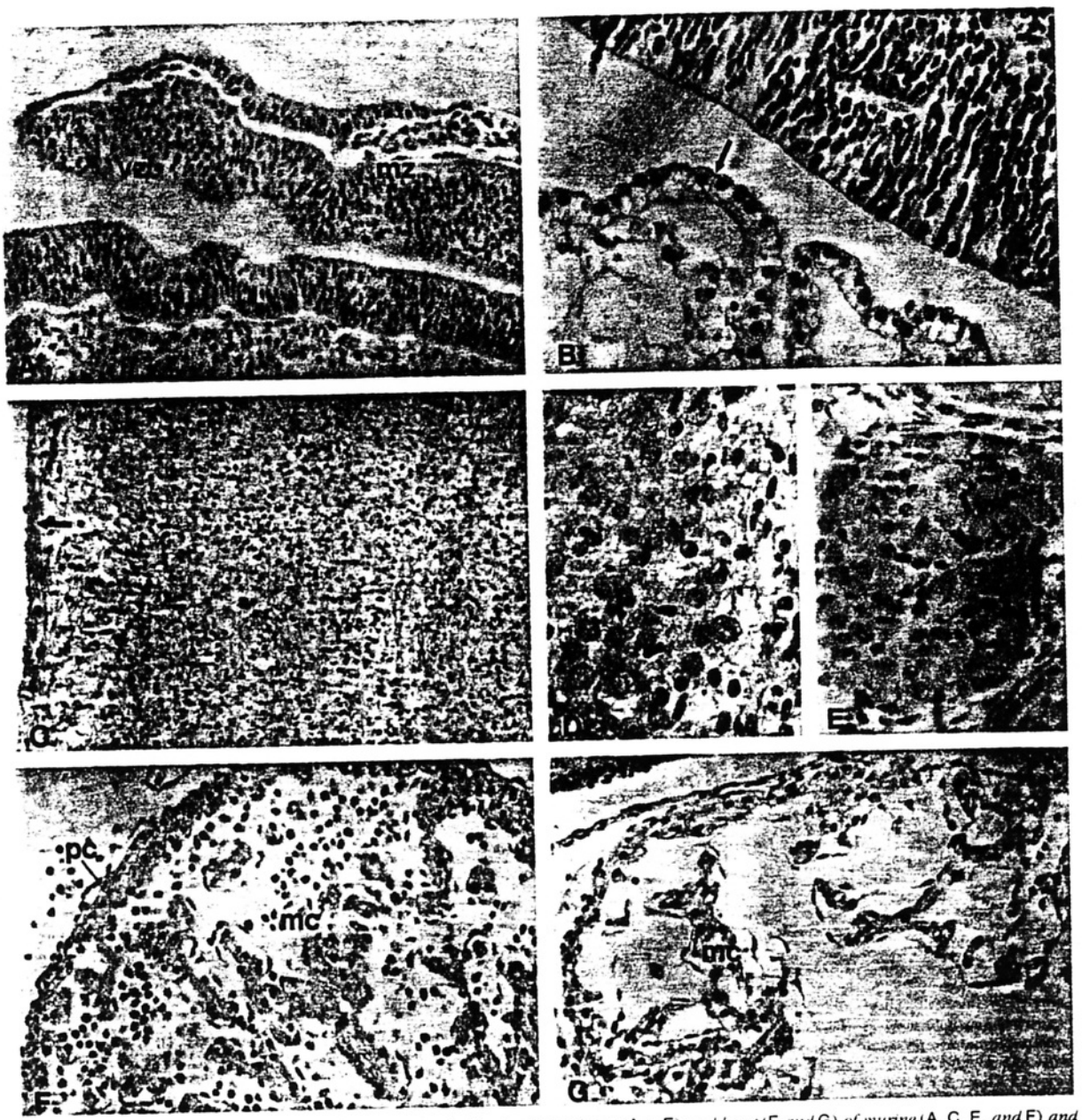


Figure 3. *Immunohistochemical detection of TF expression in the developing brain* (A *to* E) *and heart* (F *and* G) *of murine* (A, C, E, *and* F) *and human* (B, D, *and* G) *embryos* (*immunoperoxidase procedure*). A: *Section of a murine neural tube* (*8.5 days pc*). *vz, ventricular zone*; *mz, marginal zone*. B: *Human fetal brain* (*11 weeks*). Arrow *indicates choroid plexus*. C: *Section of murine embryonic mesencephalon* (*13.5 days pc*). Arrow *shows leptomeninges*. D: *Cranial nerve nucleus of a human fetus* (*11 weeks*) E: *Murine dorsal root ganglia* (*14.5 days pc*). F *and* G: *Sections of murine and human* (*stage 12*) *heart anlage, respectively*. *mc, myocardium*; *pc, pericardium*. *Magnification*, ×*190* (A, C, F, *and* G) *and* ×*380* (*9.5 days pc*) (B, D, *and* E).

and liver (Figures 4E and 5E and Table 1). Moreover, in both species, a particularly prominent expression of TF was demonstrated in the exocrine cells during pancreatic development (Figure 4, F–H, and Figure 5F).

Species-Specific TF Expression in Kidneys

In agreement with previous studies, we found that TF was expressed in the epithelia of Bowman's capsule in the adult human kidney TF (Figure 6A)[5,6,10] In contrast, in the adult murine kidney, only tubules were positive for TF (Figure 6B). Development of the urinary system depends on directed growth and mutual induction of separate anlage, the ureteric bud and the metanephrogenic blastema. In renal development, an initially weak expression of TF was detected in both murine and human tubuli formed by the proliferating ureteric bud. The blind-ended arches of these tubuli are known to induce the cells

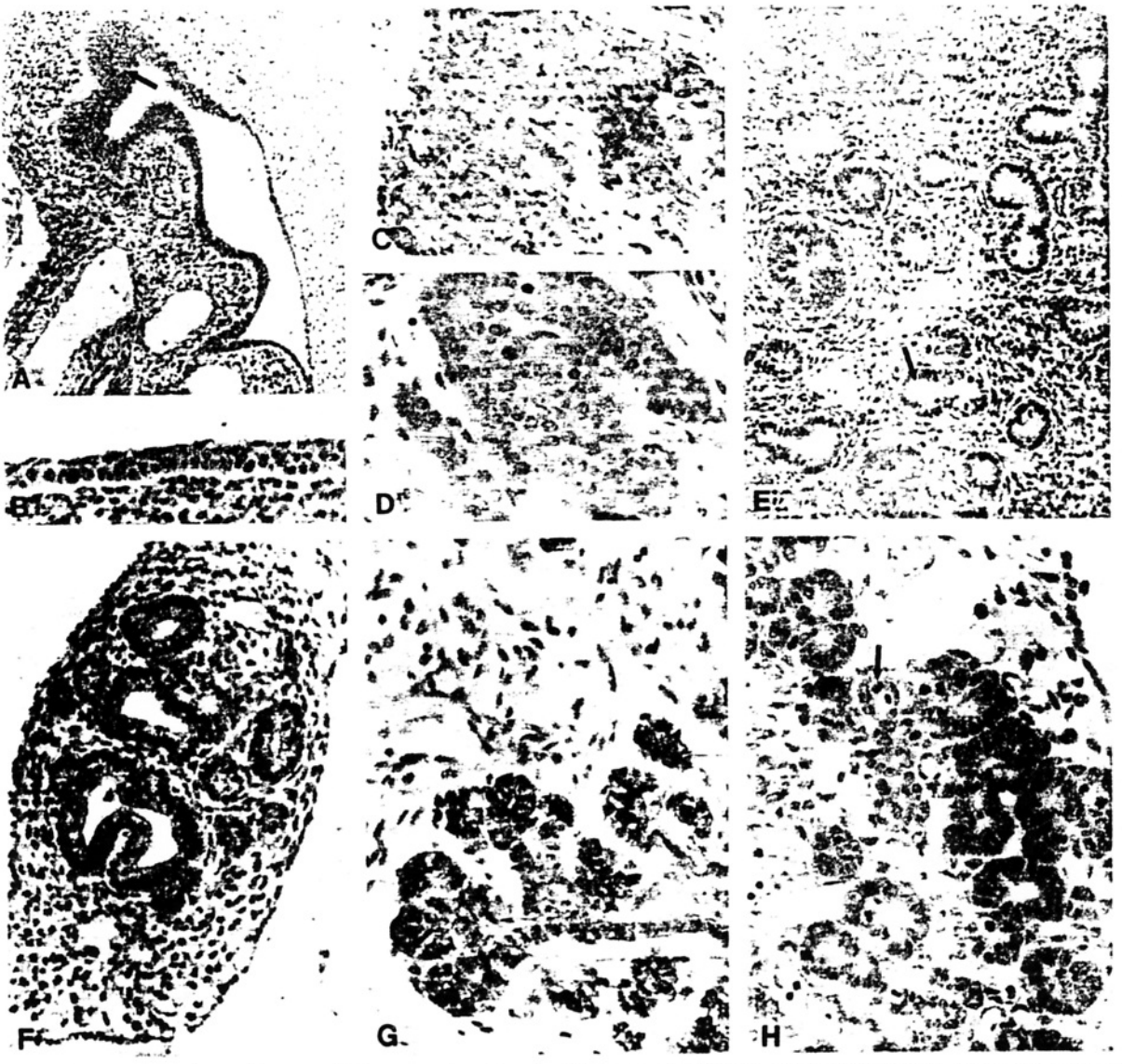


Figure 4. *Immunohistochemical detection of TF expression in embryonic epithelia of human* (A, C, F, *and* G) *and murine* (B, D, E, *and* H) *embryos* (*immunoperoxidase procedure*). A: *Sagittal section of human embryonic stomodeum* (*stage 12*). Arrow *indicates epithelium positive for TF.* B: *Murine skin* (*14.5 days pc*). C and D: *Localization of TF in the human* (*11 weeks*) *and murine* (*15.5 days pc*) *adenohypophysis anlage, respectively.* E: *Murine lung* (*15.5 days pc*). Arrow *shows developing bronchus.* F *and* G: *TF expression in exocrine cells of the human embryonic* (*stage 22*) *and fetal* (*11 weeks*) *pancreas, respectively.* H: *Murine pancreas* (*18.5 days pc*). Arrow *shows exocrine cells. Magnification,* ×*95* (A), ×*190* (C, E, *and* F), *and* ×*380* (B, D, G, *and* H).

of the metanephrogenic blastema to form the glomeruli. At this stage of renal development, strong expression of TF in human maturing glomeruli was detectable (Figure 6C). In mice, however, glomerular structures remained negative whereas a moderate to strong expression of TF mRNA and protein was detectable in distinct tubular segments during kidney development (Figures 5G and 6D).

Discussion

From the distinct and selective pattern of cellular expression of TF observed in adult tissues, it was presumed that the *in vivo* cellular expression of TF was determined by differentiation during embryogenesis.[2,5,6] Using immunohistochemistry and *in situ* hybridization, we could detect expression of TF during both mouse and human ontogenetic development. The widespread distribution of TF in embryos as well as in adult species suggests that TF might have functions in addition to that of a receptor for coagulation factor VII and an initiator of the coagulation cascade.[2,5,30,38] From this point of view, abundant TF expression at early human embryogenesis (stage 5 to 12) before detectable protein expression of factor VII seems to be most important. This obser-

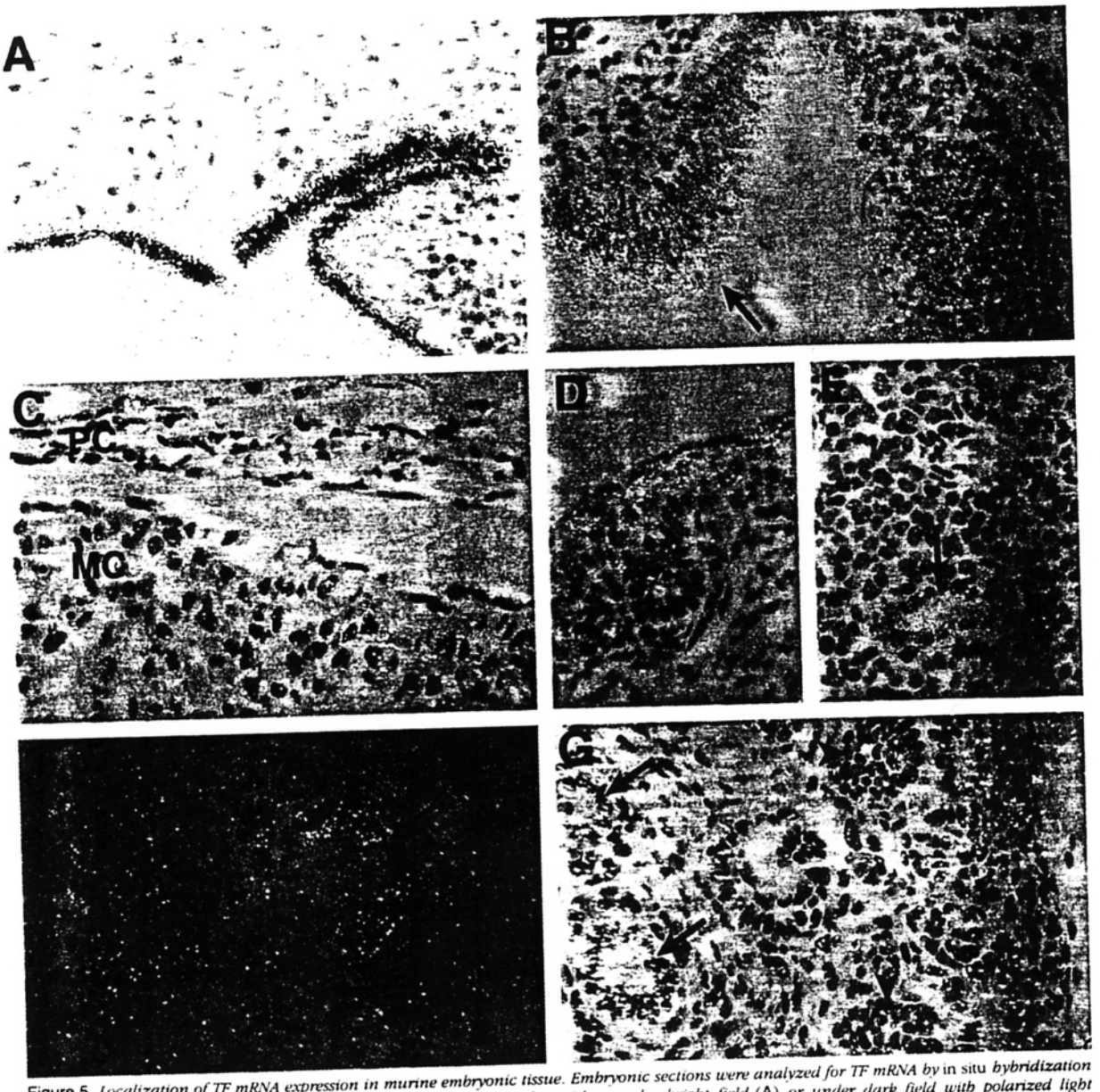


Figure 5. *Localization of TF mRNA expression in murine embryonic tissue. Embryonic sections were analyzed for TF mRNA by* in situ *hybridization as described in Materials and Methods. Photomicrographs were taken either under bright field* (A) *or under dark field with polarized light epiluminescence* (B *to* G). *Positive hybridization signals appear as black grains (bright field) or white-green grains (epiluminescence).* A: *Stomodeum (11.5 days pc).* B: *Embryonic brain (15.5 days pc).* Arrow *indicates ventricular wall.* C: *Heart anlage (12.5 days pc). PC, pericardium; MC, myocardium.* D: *Embryonic skin (15.5 days pc).* E: *Lung (15.5 days pc).* Arrow *shows developing bronchus.* F: *Pancreas anlage (14.4 days pc).* Arrow *indicates exocrine cells.* G: *Embryonic kidney (14.5 days pc).* Arrow *shows tubular epithelium and* arrowhead *shows developing glomerulus. Magnification, ×400* (A *to* G).

vation by immunhistochemistry is consistent with the finding that the vitamin-K-dependent clotting factors including factor VII were detected first in the late embryonic period or pre-viable fetal period, respectively.[39,40] Because maternal coagulation factors should not freely pass the placenta at this stage of the embryonic period of human development,[30] blood samples from embryos do not clot in whole blood clotting time experiments.[41] Activity in the extrinsic coagulation pathway measured by clotting tests has been demonstrated as early as 7 to 10 weeks of gestational age.[39,40] Although we cannot absolutely exclude minor expression of factor VII with regard to the detection limit of immunohistochemistry, the absence of detectable amounts of factor VII compared with the broad distribution of TF at early developmental stages might indicate that other distinct proteases or peptide ligands for TF exist. This is similar to other receptors of the coagulation cascade such as the thrombin receptor and

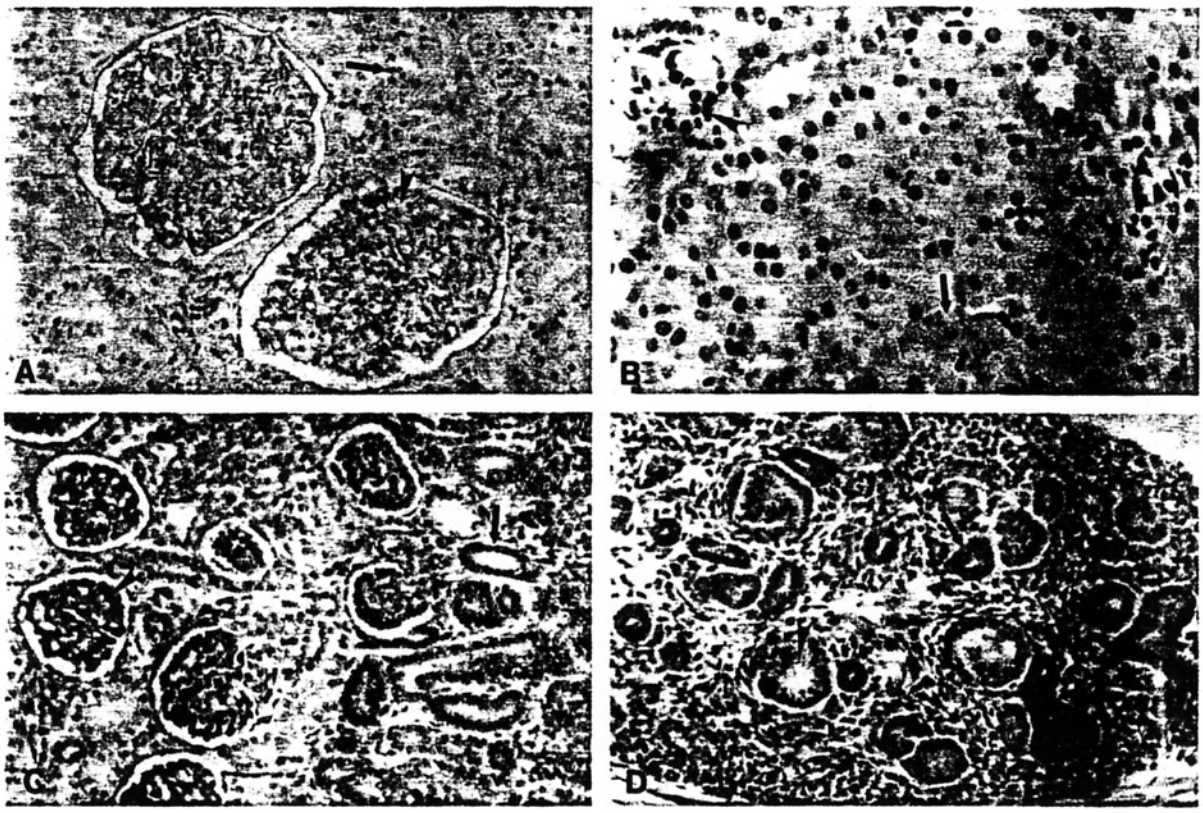


Figure 6. *Immunohistochemical detection of TF expression in adult* (A *and* B) *and embryonic* (C *and* D) *kidneys of human* (A *and* C) *and murine* (B *and* D) *species* (*immunoperoxidase procedure*). A: *Parietal and visceral epithelia of glomeruli in the human adult kidney stained for TF.* B: *In the murine adult kidney, only tubular epithelia were stained for TF.* Arrows *show tubular epithelia and* arrowheads *show glomeruli* (A *and* B). C: *Human fetal kidney* (*17 weeks*). D: *Murine embryonic kidney* (*13.5 days pc*). Arrows *show tubular epithelia and* arrowheads *show developing glomeruli* (C *and* D). *Magnification.* ×*190* (A, C, *and* D) *and* ×*380* (B).

thrombomodulin, which are abundantly expressed in normal embryonic development before the physiological ligand is detectable.[30,42] In the case of thrombomodulin, it could be demonstrated that the receptor is necessary for normal embryonic development *in utero* and its absence is not compatible with survival.[43]

During embryogenesis, the cellular localization of TF was not restricted to the cell membrane. Intracellular staining for TF, especially in more undifferentiated cells, was obvious in primary ectodermal and entodermal cells. This observation is consistent with previously published data showing that TF expression could be detected in a perinuclear location in various cells including smooth muscle cells and tumor cells.[38,44] As recently described, the optimal cell surface expression of TF is partly dependent on glycosylation.[45] Thus, the highly variable glycosylation pattern of TF from different tissues,[46] which seems to be dependent on the phenotypic differentiation level of the cells, may indicate that the predominant intracellular detectable TF is not optimal expressed on the cell surface during early developmental stages.[45–47] Because disruption of the TF gene leads to embryonic lethality,[28] this observation emphasizes that, in the absence of detectable amounts of factor VII, intracellular expressed TF could play an additional role beside its known function as membrane receptor during early development and malignancy.

In both mouse and human organogenesis, there was a striking early expression of TF in the cardiovascular and central nervous systems. This result is indicative of a possible role of TF in the early development of these tissues as described for other protease receptors.[30,43] In addition to an early expression of TF in undifferentiated neuroepithelial cells of the brain anlage, we could demonstrate a moderate to strong staining reaction in primordial glial cells as well as in early neuronal cells. In contrast, in the adult brain, astrocytes were described as the primary source of TF.[8,10] The pattern of early expression of TF in the embryonic nervous system suggests a possible role of TF in brain development, which seems to be different from its role in the adult organism. The significance of TF expression in the adeno-

hypophysis anlage, which might be of neuroectodermal rather than ectodermal (stomodeal) origin, is still unclear,[48-50] although we found a particularly prominent expression of TF in the endocrine cells of the adult pituitary gland (M. Kasper, unpublished observation).

In addition to neuronal cells, there was also robust TF expression in other tissues, from which TF is absent in the adult counterpart. Thus, we found a strong expression in the exocrine cells during pancreatic development. In the adult human pancreas, epithelia were normally negative and only expressed TF in acute tryptic inflammation (M. Müller, unpublished observation) or during neoplastic transformation.[51]

In both species, the expression of TF during ontogenetic development was observed in tissues known to have a constitutive expression of this protein in the adult organism, such as epidermis, myocardium, brain, bronchial epithelium, and hepatocytes. With respect to kidney, constitutive TF expression differs in humans and mice. In human kidneys, epithelia of both layers of Bowman's capsule expressed TF strongly,[5,6,10] whereas during nephrogenesis and in adult murine kidneys, the epithelia of some tubular segments were positive for TF in the absence of staining of the glomeruli.[52] The cause of the difference is still unknown, although this might indicate histophysiological differences in glomerular structure between humans and mice.

We have demonstrated the localization of TF during ontogenetic development. The abundance of TF in early development before detectable expression of factor VII supports our contention that TF may be an important morphogenic factor in embryogenesis.

Acknowledgments

We thank Ms. I. Peterson, Ms. S. Thieme, Ms. H. Rilke, Ms. S. Langer, and Ms. H. Riester for excellent technical support.

References

1. Nemerson Y, Bach R: Tissue factor revisited. Prog Hemost Thromb 1982, 6:237–261
2. Edgington TS, Mackman N, Brand K, Ruf W: The structural biology of expression and function of tissue factor. Thromb Haemost 1991, 66:67–79
3. Williams WJ: The activity of lung microsomes in blood coagulation. J Biol Chem 1964, 239:933–942
4. Williams WJ: The activity of human placenta microsomes and brain particles in blood coagulation. J Biol Chem 1966, 241:1840–1846
5. Drake TA, Morrissey JH, Edgington TS: Selective cellular expression of tissue factor in human tissues. Am J Pathol 1989, 134:1087–1097
6. Fleck RA, Rao LVM, Rapaport SI, Varki N: Localization of human tissue factor antigen by immunostaining with monospecific, polyclonal anti-human tissue factor antibody. Thromb Res 1990, 59:421–437
7. del Zoppo GJ, Yu J-Q, Copeland BR, Thomas WS, Schneiderman J, Morrissey JH: Tissue factor localization in non-human primate cerebral tissue. Thromb Haemost 1992, 68:642–647
8. Eddleston M, de la Torre JC, Oldstone MBA, Loskutoff DJ, Edgington TS, Mackman N: Astrocytes are the primary source of tissue factor in the murine central nervous system. J Clin Invest 1993, 92:349–358
9. Mackman N, Sawdey MS, Keeton MR, Loskutoff DJ: Murine tissue factor gene expression *in vivo*. Tissue and cell specificity and regulation by lipopolysaccharide. Am J Pathol 1993, 143:76–84
10. Flössel C, Luther T, Müller M, Albrecht S, Kasper M: Immunohistochemical detection of tissue factor (TF) on paraffin sections of routinely fixed human tissue. Histochemistry 1994, 101:449–453
11. Mackman N: Regulation of tissue factor gene. FASEB J 1995, 9:883–889
12. Rao LVM, Rapaport SI: Activation of factor VII bound to tissue factor: a key early step in the tissue factor pathway of blood coagulation. Proc Natl Acad Sci USA 1988, 85:6687–6691
13. Hartzell S, Ryder K, Lanahan A, Lau LF, Nathans D: A growth factor-responsive gene of murine Balb/c 3T3 cells encodes a protein homologous to human tissue factor. Mol Cell Biol 1989, 9:2567–2573
14. Ranganathan G, Blatti SP, Subramanian M, Fass DN, Maihle NJ, Getz MJ: Cloning of murine tissue factor and regulation of gene expression by transforming growth factor type β1. J Biol Chem 1991, 266:496–501
15. Edwards RL, Rickles FR, Bobrove AM: Mononuclear cell tissue factor: cell of origin and requirements for activation. Blood 1979, 54:359–370
16. Mackman N, Fowler BJ, Edgington TS, Morrissey JH: Functional analysis of the human tissue factor promoter and induction by serum. Proc Natl Acad Sci USA 1990, 87:2254–2258
17. Luther T, Flössel C, Hietschold V, Koslowski R, Müller M: Flow cytometric analysis of tissue factor (TF) expression on stimulated monocytes: comparison to procoagulant activity of mononuclear blood cells. Blut 1990, 61:375–378
18. Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone MA: Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. J Exp Med 1984, 160:618–623
19. Gregory SA, Edgington TS: Tissue factor induction in human monocytes: two distinct mechanisms displayed

by different alloantigen responsive T cell clones. J Clin Invest 1985, 76:2440–2445
20. Nawroth PP, Stern DM: Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J Exp Med 1989, 163:740–745
21. Bierhaus A, Zhang Y, Deng Y, Mackman N, Quehenberger P, Haase M, Luther T, Müller M, Böhrer M, Greten J, Martin E, Baeuerle PA, Waldherr R, Kisiel W, Ziegler R, Stern DM, Nawroth PP: Mechanism of the TNFα mediated induction of endothelial tissue factor. J Biol Chem 1995, 270:26419–26432
22. Parry GC, Mackman N: Transcriptional regulation of tissue factor expression in human endothelial cells. Arterioscler Thromb Vasc Biol 1995, 15:612–621
23. Drake TA, Cheng J, Chang A, Taylor FB Jr: Expression of tissue factor, thrombomodulin and E-selectin in baboons with lethal *Escherichia coli* sepsis. Am J Pathol 1993, 142:1458–1470
24. Flössel C, Luther T, Albrecht S, Kotzsch M, Müller M: Constitutive tissue factor expression of human breast cancer cell line MCF-7 is modulated by growth factors. Eur J Cancer 1992, 28A:1999–2002
25. Lockwood CJ, Nemerson Y, Guller S, Krikun G, Alvarez M, Hausknecht V, Gurpide E, Schatz F: Progestational regulation of human endometrial stromal cell tissue factor expression during decidualization. J Clin Endocrinol Metab 1993, 76:231–236
26. Sturm U, Luther T, Albrecht S, Flössel C, Grossmann H, Müller M: Immunohistological detection of tissue factor in normal and abnormal human mammary glands using monoclonal antibodies. Virchows Arch A Pathol Anat 1992, 421:79–86
27. Carson SD, Henry WM, Shows TB: Tissue factor gene localized to human chromosome 1 (1pter-1p21). Science 1985, 229:991–993
28. Carmeliet P, Mackman N, Wyns S, Kieckens L, Edgington T, Collen D: Inactivation of the tissue factor gene in embryonic stem cells. Thromb Haemost 1995, 73: A1059
29. Flössel C, Luther T, Albrecht S, Müller M: Tissue factor expression during human ontogenetic development. Ann Hematol 1995 70:A76
30. Soifer SJ, Peters KG, O'Keefe J, Coughlin SR: Disparate temporal expression of the prothrombin and thrombin receptor genes during mouse development. Am J Pathol 1994, 144:60–69
31. Zhang Y, Deng Y, Luther T, Müller M, Ziegler R, Waldherr R, Stern DM, Nawroth PP: Tissue factor controls the balance of angiogenic and antiangiogenic properties of tumor cells in mice. J Clin Invest 1994, 94:1320–1327
32. Albrecht S, Luther T, Grossmann H, Flössel C, Kotzsch M: An ELISA for tissue factor using monoclonal antibodies. Blood Coagul Fibrinol 1992, 3:263–270
33. Albrecht S, Müller S, Siegert G, Luther T, Müller M: Plasma concentration of tissue factor and factor VII in patients after abdominal surgery. Thromb Haemost 1995, 77:557–562
34. Kasper M, Schuh D, Müller M: Immunohistochemical localization of the β-subunit of prolyl 4-hydroxylase in human alveolar epithelial cells. Acta Histochem 1994, 96:309–313
35. Luther T, Bierhaus A, Kasper M, Kotzsch M, Flössel C, Zhang Y, Albrecht S, Grosser M, Grossmann H, Nawroth PP, Müller M: Activation of monocytes by endotoxin *in vivo*. Thromb Haemost 1995, 73:A1082
36. Mackman N, Imes S, Maske WH, Taylor B, Lusis AJ, Drake TA: Structure of the murine tissue factor gene: chromosome location and conservation of regulatory elements in the promoter. Arterioscler Thromb 1992, 12:474–483
37. DeRuiter MC, Poelmann RE, VanderPlas-de Vries I, Mentnik MMT, Gittenberger de Groot AC: The development of the myocardium and endocardium in mouse embryos. Anat Embryol 1992, 185:461–473
38. Müller M, Flössel C, Haase M, Luther T, Albrecht S, Nawroth PP, Zhang Y: Cellular localization of tissue factor in human breast cancer cell lines. Virchows Arch B Cell Pathol 1993, 64:265–269
39. Hassan HJ, Leonardi A, Chelucci C, Mattia G, Macioce G, Guerriero R, Russo G, Mannucci PM, Peschle C: Blood coagulation factors in human embryonic-fetal development: preferential expression of the FVII/tissue factor pathway. Blood 1990, 76:1158–1164
40. Wadsworth LD, Massing BG, Mitchell L: Hemostatic, hematopoietic, and immune systems. Developmental Pathology of the Embryo and Fetus. Edited by JE Dimmick, DK Kalousek, Philadelphia, JB Lippincott, 1992, pp 749–798
41. Zilliacus H, Ottelin AM, Mattson T: Blood clotting and fibrinolysis in human foetuses. Biol Neonat 1966, 10: 108–113
42. Imada S, Yamaguchi H, Nagumo M, Katayanagi S, Iwasaki H, Imada M: Identification of fetomodulin, a surface marker protein of fetal development, as thrombomodulin by gene cloning and functional assays. Dev Biol 1990, 140:113–122
43. Healy AM, Rayburn HB, Rosenberg RD, Weiler H: Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. Proc Natl Acad Sci USA 1995, 92:850–854
44. Nemerson Y: Tissue factor: then and now. Thromb Haemost 1995, 74:180–184
45. Rickles FR, Hair GA, Zeff RA, Lee E, Bona RD: Tissue factor expression in human leukocytes and tumor cells. Thromb Haemost 1995, 74:391–395
46. van der Besselaar AMHP, Bertina RM: Interaction of thromboplastin apoprotein of different tissues with concanavalin A: evidence for heterogenous glycosylation of the human apoprotein. Thromb Haemost 1984, 52: 192–195
47. Fenderson BA, Andrews PW: Carbohydrate antigens of embryonal carcinoma cells: changes upon differentiation. APMIS Suppl 1992, 27:109–118
48. Takor T, Pearse AGE: Neuroectodermal origin of ovar-

ian hypothalamo-hypophyseal complex: the role of the ventral neural ridge. J Embryol Exp Morphol 1975, 34:311–325
49. Gluckman PD: The fetal neuroendocrine axis. Curr Top Exp Endocrinol 1983, 5:1–8
50. Kasper M, Stosiek P, van Muijen GNP, Moll R: Cell type heterogeneity of intermediate filament expression of the human pituitary gland. Histochemistry 1989, 93:93–10
51. Silberberg JM, Gordon S, Zucker S: Identification of tissue factor in two human pancreatic cancer cell lines. Cancer Res 1989, 49:5443–5447
52. Pemberton KD, Tuddenham EGD, McVey JH: Tissue localization of murine tissue factor (TF) antigen by immunostaining with a polyclonal chicken anti-human TF antibody. Thromb Haemost 1995, 73:A1073