Polyoma Middle T-induced Vascular Tumor Formation: The Role of the Plasminogen Activator/Plasmin System

Kanaga T. Sabapathy,* Michael S. Pepper,§ Friedemann Kiefer,‡ Uta Möhle-Steinlein,* Fabienne Tacchini-Cottier,‖ Ingrid Fetka,* Georg Breier,¶ Werner Risau,¶ Peter Carmeliet, Roberto Montesano,§ and Erwin F. Wagner***

*Research Institute of Molecular Pathology, University of Vienna, A-1030 Vienna, Austria; ‡Ontario Cancer Institute, Toronto, Ontario, Canada; §Department of Morphology, ‖Department of Pathology, University Medical Center, CH-1211 Geneva 4, Switzerland; ¶Max-Planck Institute for Physiological and Clinical Research, Department of Molecular and Cell Biology, D-61231 Bad Nauheim, Germany; and **Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, B-3000 Leuven, Belgium

Abstract. The middle T antigen of murine Polyomavirus (PymT) rapidly transforms endothelial cells, leading to the formation of vascular tumors in newborn mice. Transformed endothelial (End.) cell lines established from such tumors exhibit altered proteolytic activity as a result of increased expression of urokinase-type plasminogen activator (uPA) and are capable of inducing vascular tumors efficiently when injected into adult mice. In this study we have used mice lacking components of the PA/plasmin system to analyze the role of this system in the transformation process and in tumor growth. We found that the proteolytic status of the host is not a critical determinant for PymT-induced vascular tumor formation. In addition, the lack of either uPA or tissue-type PA (tPA) activity is not limiting for the establishment and proliferation of End. cells in vitro, although the combined loss of both PA activities leads to a marked reduction in proliferation rates. Furthermore, the in vitro morphogenetic properties of mutant End. cells in fibrin gels could only be correlated with an altered proteolytic status in cells lacking both uPA and tPA. However, in contrast with tumors induced by PymT itself, the tumorigenic potential of mutant and wild-type End. cell lines was found to be highly dependent on the proteolytic status of both the tumor cells and the host. Thus, genetic alterations in the PA/plasmin system affect vascular tumor development, indicating that this system is a causal component in PymT-mediated oncogenesis.

Oncogenesis is a multistep process that requires a long latency period between the initiating event and the appearance of the tumor. However, a few viral oncogenes are known to rapidly transform target tissues without the need for additional genetic events; one such viral oncogene is the Polyoma middle T antigen (PymT).[1] The PymT antigen specifically transforms proliferating endothelial cells apparently in a single-step manner, resulting in the formation of hemorrhagic cystic tumors in embryonic and neonatal mice, but not in adult mice where endothelial cell proliferation has ceased (for review see Pepper et al., 1997). The potency with which PymT transforms endothelial cells is thought to be a consequence of its interaction with proteins of the signal transduction machinery (for review see Brizuela et al., 1994; Kiefer et al., 1994*a*). These tumors are functionally reminiscent of human vascular tumors in that they are endothelial specific and organ nonspecific (for review see Wagner and Risau, 1994; Pepper et al., 1997), and that tumor-bearing mice develop features of the Kasabach-Merritt syndrome, i.e., thrombocytopenia, anemia, and associated splenomegaly (Dubois-Stringfellow et al., 1994*a*).

Tumor formation and subsequent invasion involve the disruption of anatomical barriers including basement membranes and penetration of tumor cells into normal adjacent tissues. Several lines of evidence indicate that enhanced invasive and metastatic potential is correlated with increased protease activity. It has been shown that both tumor and stromal cells express enzymes that degrade components of the extracellular matrix. Many of the relevant enzymes belong to one of two families: the serine proteases, in particular the plasminogen activator (PA)/plasmin

Address all correspondence to Erwin F. Wagner, Research Institute of Molecular Pathology, University of Vienna, A-1030 Vienna, Austria. Tel.: 43-1-798-7153. Fax: 43-1-797-30-630.

1. *Abbreviations used in this paper*: End., endothelioma; MMP, matrix metalloproteinase; PA, plasminogen activator; uPA, urokinase-type PA; uPAR, uPA receptor; tPA, tissue-type PA; PAI, PA inhibitor; Plg, plasminogen; PTAH, phosphotungstic acid–hemotoxylin; PymT, Polyoma middle T antigen.

system, and the matrix metalloproteinases (MMPs). Urokinase and tissue-type PAs (uPA and tPA) are the principal activators of plasminogen (Plg), the zymogen from which plasmin is derived. uPA is secreted in the form of an inactive precursor that binds with high affinity to a specific glycosylphosphatidylinositol-anchored cell surface receptor. Plasmin is a protease of tryptic specificity that either directly, or indirectly through the activation of latent MMPs, hydrolyzes extracellular proteins. The existence of multiple specific physiological inhibitors of both plasmin, i.e., α_2-antiplasmin, and PAs, i.e., PA inhibitors 1 and 2 (PAI-1 and PAI-2), provides additional points of regulation along this protease cascade (for review see Danø et al., 1985; Vassalli et al., 1991; Pepper et al., 1996).

The recent advent of mice lacking uPA, uPA receptor (uPAR), tPA, PAI-1, and Plg has questioned much early dogma concerning the role of the PA/plasmin system in processes requiring extracellular matrix breakdown. Thus, uPA−/−, tPA−/−, and uPA−/− and tPA−/−(utPA−/−) mice, as well as Plg−/− mice, develop normally in utero and are fertile. However, utPA−/− mice develop multiple organ failure starting at 2–3 mo of age as a result of extensive extravascular fibrin accumulation as well as generalized microvascular thrombosis (Carmeliet et al., 1994). utPA−/− mice thus have retarded postnatal growth, exhibit reduced fertility, and have a shortened life span. A similar phenotype is observed in Plg-deficient mice (Bugge et al., 1995*a*; Ploplis et al., 1995). The phenotype of both utPA−/− and Plg-deficient mice therefore appears to be due to a systemic defect in fibrinolysis. Although no overt phenotypic alterations have been observed in uPAR−/− mice with respect to development, fertility, and homeostasis (Bugge et al., 1995*b*), it has recently been reported that adult mice with a combined deficiency in tPA and uPAR display hepatic sinusoidal fibrin deposits, demonstrating that uPAR plays a role in physiological fibrinolysis (Bugge et al., 1996). Finally, development and fertility are unaffected in PAI-1–deficient mice, although the absence of PAI-1 induces a mild hyperfibrinolytic state (Carmeliet et al., 1993*a,b*). Therefore, neither PAs nor Plg are essential for mouse development, postnatal growth, or fertility, suggesting that the primary role for the PA/plasmin system in the mouse is fibrinolysis.

In previous studies with PymT-transformed endothelial (End.) cell lines in a fibrin gel system, we observed that End. cells formed cystlike structures rather than capillary-like tubes, which correlated with their increased proteolytic activity (Montesano et al., 1990). Reduction of protease activity by the addition of broad spectrum serine protease inhibitors led to the formation of capillary-like tubes, resulting in the hypothesis that tightly controlled extracellular proteolysis is essential for normal capillary morphogenesis (Pepper and Montesano, 1990). Further support for the role of the PA/plasmin system in aberrant morphogenesis in vitro came from experiments in which depletion of Plg from serum or addition of neutralizing antibodies to murine uPA inhibited cyst formation (Dubois-Stringfellow et al., 1994*b*). These findings implicated increased proteolysis, and in particular increased uPA activity, in aberrant vascular morphogenesis in vitro and further suggested a causal role for PymT in the elevated proteolytic activity of End. cells. In this study, mice lacking uPA, tPA, utPA, PAI-1, or Plg were used to investigate whether the PA/plasmin system might be important for PymT- or End. cell–induced tumor formation.

Materials and Methods

Mice

The generation of uPA−/−, tPA−/−, uPA,tPA−/− (utPA−/−), PAI-1−/−, and Plg−/− mice has been described (Carmeliet et al., 1993*a*, 1994; Ploplis et al., 1995). All mice in a C57Bl6/129 background were propagated by brother–sister matings and have been interbred for more than six generations. CD1 nude mice were purchased from CharlesRiver (Sulzfeld, Germany).

Virus Induced Tumor Induction in Neonatal Mice

Helper-free stocks of the N-TKmT virus were obtained from the viral producer cell line GP+E subclone 108.4.2 as described previously (Williams et al., 1988). Neonatal mice of various genotypes were injected intraperitoneally with 1–2.5 × 10^5 neo^R-transferring particles. The mice were observed carefully and analyzed as soon as the first mouse of an injected litter showed signs of anemia.

Derivation and Maintenance of End. Cells

Macroscopically visible tumors from mice injected with PymT viral particles were explanted, and End. cell lines were established as previously described (Kiefer et al., 1994*b*). End. cells could be established with relative ease from heterogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing, mostly clonal population of End. cells was obtained, which was used for the experiments described. Cell lines were maintained in DME supplemented with 5% FCS, glutamine, and nonessential amino acids and subcultured at confluence every 4–8 d. The origin and nomenclature of these cell lines are summarized in Table I.

Determination of Proliferation Rates of End. Cells

End. cells were plated at a density of 4 × 10^5–1 × 10^6 per 25-cm^2 flask, counted, and replated at the same density every 6–8 d. Total cumulative cell numbers are represented over 23 d in culture.

Tumor Induction by Injection of End. Cells in Adult Mice

For the induction of End. cell–induced tumors, C57Bl6/129 wild-type and mutant mice, derived from brother–sister matings and interbred for more than six generations (10–14 wk old), and CD1 nude mice (8–10 wk old) were injected subcutaneously into the flanks of the right leg with 1.5–2 × 10^6 cells, and then scored for tumor formation. The times taken for the onset of tumors and the latency period to form tumors of 125 mm^2 (maximum size) were recorded. All mice were killed when they had 125-mm^2 tumors. The observation period was until 60 d after injection.

Northern Blot Hybridization

RNA analyses were carried out using standard Northern techniques (Kiefer et al., 1994*b*). The probes were kindly provided by D. Belin (University of Geneva, Switzerland) (uPA), S. Strickland (State University of New York, Stony Brook) (tPA), and R. Bravo (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ) (PAI-1).

Immunohistochemical Analysis and Immunocytochemistry

Tissue specimens were explanted, dissected, fixed overnight in 4% paraformaldehyde in PBS, and embedded in paraffin; 5-μm-thick sections were stained with hematoxylin and eosin. Adjacent sections were used for immunocytochemical reactions with antibody against factor VIII–related antigen/von Willebrand factor (vWF) (Dakopatts, Copenhagen, Den-

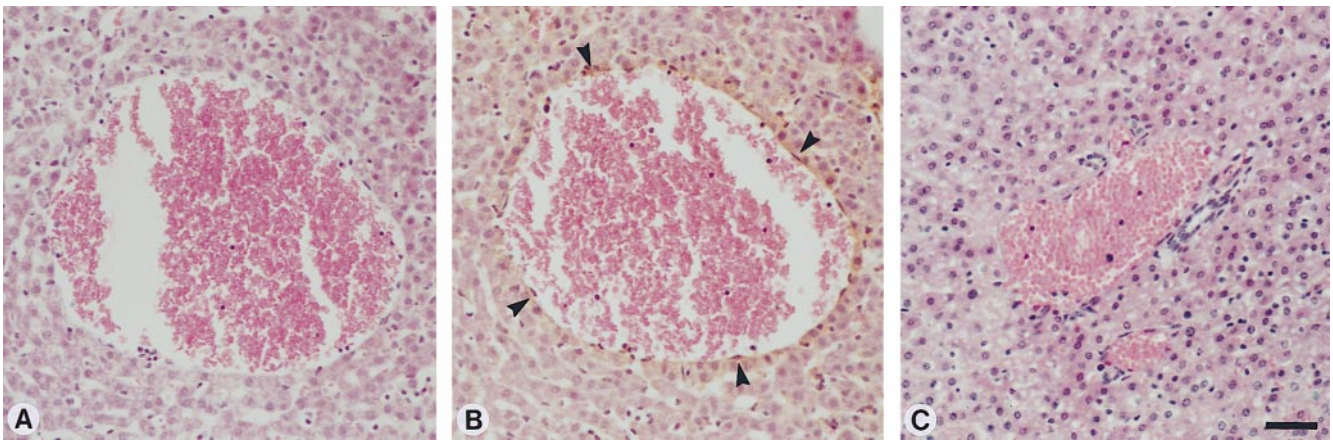


Figure 1. PymT virus–induced vascular tumors in newborn mice lacking utPA (*A* and *B*) and Plg (*C*). H/E (*A* and *C*) and vWF (*B*) staining is shown. (*Arrowheads*) Endothelial cells lining the tumors. Bar, 50 μm.

mark). Some sections were processed using special staining techniques for fibrin. These included the Lendrum (Lendrum et al., 1962) and Mallory's phosphotungstic acid–hematoxylin (PTAH) techniques.

Zymography and Reverse Zymography

Confluent monolayers of End. cells in 35-mm tissue-culture dishes were washed twice with serum-free DME, and 1.5 ml serum-free DME containing 200 KIU/ml Trasylol (Bayer AG, Zurich, Switzerland) was added. 15 h later, cell extracts and culture supernatants were prepared and analyzed by zymography and reverse zymography as previously described (Vassalli et al., 1984; Montesano et al., 1990). Cell number was determined in a second set of dishes processed in parallel, and cell extract and culture supernatant samples were analyzed on the basis of cell equivalents.

In Vitro Fibrin Gel Assay

End. cells were seeded in suspension into 500-μl fibrin gels at 1×10^4 cells per gel. 500 μl DME containing 10% FCS was added to each well above the fibrin gels. Fibrin gels were prepared as previously described (Montesano et al., 1990). All experiments were performed in the absence or presence of 200 KIU/ml Trasylol, which was added both to the gel and to the medium at the time of embedding. Medium (±Trasylol) was renewed every 2–3 d. Between 4 and 16 d after seeding, cultures were fixed in situ overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and photographed using a Phase Contrast-2 microscope (Nikon Inc., Garden City, NY).

Results

PymT Efficiently Induces Vascular Tumors in the Absence of Individual Components of the PA/Plasmin System

To determine if PA/plasmin-dependent proteolytic activity is an essential requirement for the formation of vascular tumors by PymT, we inoculated newborn mice lacking uPA, tPA, utPA, PAI-1, or Plg with the PymT transducing retrovirus N-TkmT (Williams et al., 1988). The frequency of tumor formation was almost 100% in all mutant mice tested, and the time required for the development of lethal tumors (latency) varied only slightly (Table I). The high penetrance in Plg−/− mice was unexpected and no delay or inhibition of tumor formation was observed. Most mice were terminally ill within 8–10 d, and only the double-mutant pups lacking uPA and tPA succumbed after a slightly longer period of 12–14 d (Table I). These utPA−/− tumors retained the characteristic morphologic properties of PymT-induced tumors (Fig. 1, *A* and *B*). All tumors appeared as blood-filled/hemorrhagic cysts lined by endothelial cells and were not different from similar tumors induced by PymT in control mice (Kiefer et al., 1994*b*). Representative sections through a typical lesion that formed in a double mutant (utPA−/−) mouse as well as in a Plg−/− mouse are shown in Fig. 1. Staining with anti-vWF antibody shows endothelial cells lining these cysts (Fig. 1 *B*). These results indicate that the PA/plasmin-dependent proteolytic status of the host is apparently not a critical determinant in the transformation of endothelial cells by PymT and in the formation of vascular tumors in newborn mice.

Table I. Vascular Tumors Induced by PymT Virus and Establishment of End. Cell Lines

Tumors				End. cell lines	
Genotype	Latency	Penetrance	Name	Abbr.	Origin
	d				
wt+/+	8–10	29/30	bEnd.1	b1	brain
			eEnd.2	e2	yolk sac
			mEnd.3	m3	mesentery
			sEnd.1	s1	skin
			sEnd.2	s2	skin thorax
uPA−/−	10–13	9/10	mEnd.u23	u23	mesentery
			sEnd.u24	u24	skin thorax
tPA−/−	11–13	8/8	mEnd.t37	t37	mesentery
			pEnd.t38	t38	pancreas
uPA−/− tPA−/−	12–14	11/11	sEnd.ut1	ut1	skin sternum
			liEnd.ut2	ut2	liver
PAI-1−/−	6–8	3/3	luEnd.p1	p1	lung
			liEnd.p3	p3	liver
Plg−/−	8–12	9/9	ND		

Supernatants from PymT-producing fibroblasts were injected into newborn wild-type and mutant mice, which were propagated by brother–sister matings. All mutant End. cell lines were established from C57Bl/6 × 129 mice. The derivation of the wild-type End. cell lines was previously described; for details regarding establishment, see Materials and Methods.

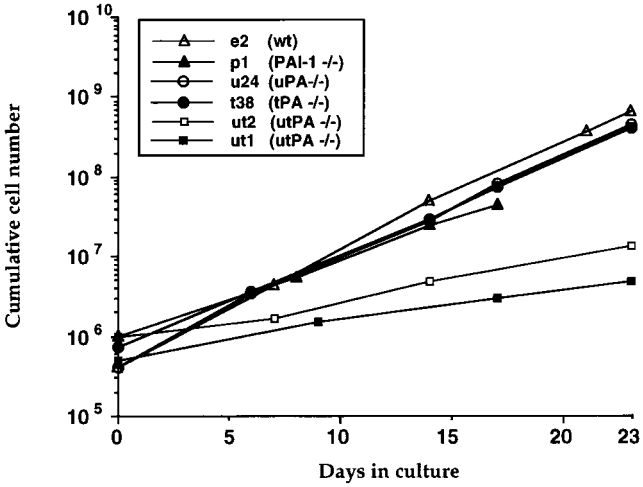


Figure 2. Proliferation rate of End. cells. The following End. cells were used for analysis of proliferation rates: e2 (*wt*), passage number 36 (p36); t38, p28 (*tPA −/−*); u24, p32 (*uPA −/−*); p1, p12 (*PAI-1 −/−*); ut1, p13 and ut2, p21 (*utPA −/−*). Total cumulative cell numbers are represented over 23 d in culture.

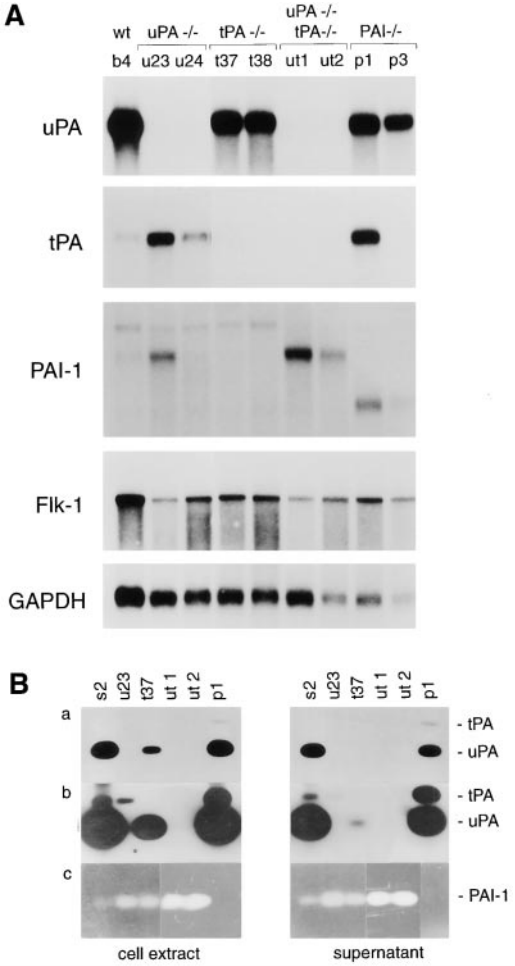


Figure 3. Expression of mRNA (*A*) and zymographic analysis (*B*) of End. cells lacking uPA, tPA, utPA, and PAI-1. (*A*) Poly A^+ RNA from the following cells were analyzed by Northern analysis: b4, passage number 41 (p41); u23 (p12); u24 (p18); t37 (p22); t38 (p16); ut1 (p9); ut2 (p11); p1 (p28); and p3 (p20). (*B*) The following End. cell extracts and culture supernatants were analyzed by zymography (*a* and *b*) and reverse zymography (*c*) as described in Materials and Methods: s2 (p29); u23 (p30); t37 (p31); ut1 (p19); ut2 (p17); and p1 (p27). (*b*) The same gel as shown in *a*, incubated at 37°C for a longer time period.

Derivation of End. Cell Lines Lacking uPA, tPA, utPA, and PAI-1

We have previously established transformed End. cell lines from tumor-bearing wild-type and various Src-kinase mutant mice, as well as by infection of primary endothelial cells by PymT in vitro (for review see Wagner and Risau, 1994; Pepper et al., 1997). To test whether the absence of components of the PA/plasmin system affects the capacity to establish permanent End. cell lines in vitro, we derived a number of independent cell lines from tumors induced in tPA-, uPA-, utPA-, and PAI-1–deficient mice (Table I). Each of these cell lines displayed the characteristic spindle-shaped, highly refractile morphology that is typical of endothelial cells (data not shown). The time required to establish the different mutant cell lines as homogeneously growing, characteristic End. cells was not significantly different using single knockout mice. However, the double mutant utPA−/− End. cells took three times as long to establish when compared with controls (data not shown). With the exception of ut1 and ut2, all End. cell lines exhibited roughly equivalent growth parameters in vitro as measured by cell doubling times after ~10 passages (Fig. 2). The proliferation rate of ut1 and ut2 cell lines was <50% of controls. All End. cell lines obtained expressed the endothelial-specific tyrosine kinase receptor Flk-1 (Fig. 3 *A*) and CD31/PECAM-1 (data not shown), confirming their endothelial origin. Most of these cell lines were clonal, since only one integration site for the provirus was detected by Southern blotting (data not shown).

To verify the genotype and measure the proteolytic

Table II. Proteolytic Activity and Morphogenetic Behavior of End. Cells Lacking uPA, tPA, utPA, and PAI-1

Genotype	Cell lines	uPA*	tPA*	PAI-1*	Behavior in fibrin gels‡
wt+/+	b1, b3, e2, s2, m1, s1	+++	++	+	Variably large to small cysts and tubes (n = 39)
uPA−/−	u23, u24	−	+	++	Medium to small cysts and tubes (n = 18)
tPA−/−	t37, t38	++	−	++	Small cysts and tubes (n = 14)
uPA−/−, tPA−/−	ut1, ut2	−	−	+++	No cysts and no tubes; isolated cells and network of cell cords (n = 6)
PAI-1−/−	p1, p3	+++	+++	−	Variably large to small cysts and network of cell cords (n = 14)

*Semiquantitative estimate determined by zymography (uPA, tPA) and reverse zymography (PAI-1).
‡Cells grown in three-dimensional fibrin gels for 4–16 d in the absence of Trasylol; n = number of experiments.
+++, high activity; ++, intermediate activity; +, low activity.

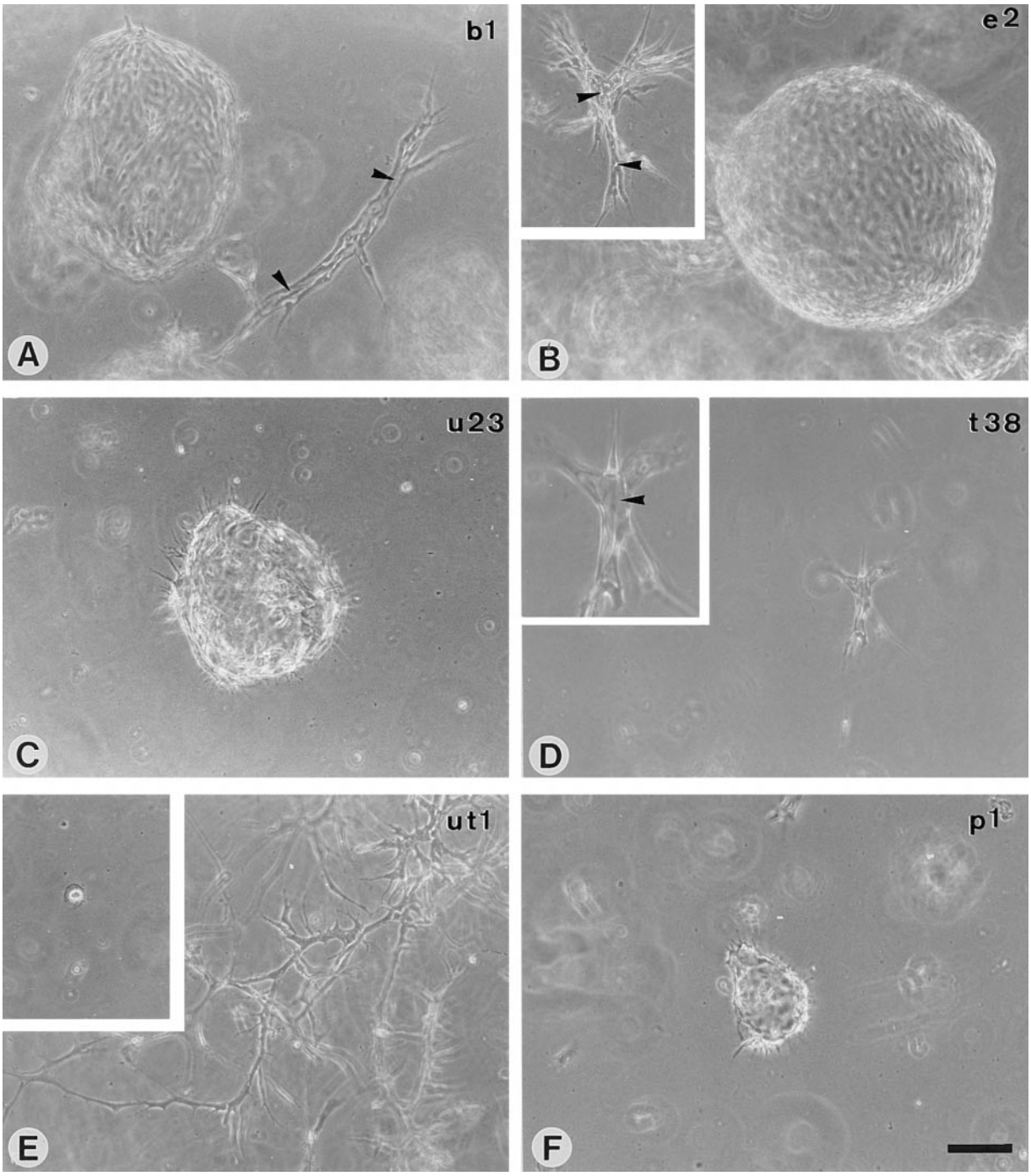


Figure 4. Morphogenetic behavior of mutant End. cells in three-dimensional fibrin gels. End. cells were seeded in suspension into three-dimensional fibrin gels and photographed by phase-contrast microscopy after the times indicated. Unless otherwise indicated, cells were grown in the absence of Trasylol. (*A*) b1 (p14) cells after 11 d in culture form a medium to large–sized cyst and a narrow tube (lumen indicated by *arrowheads*) in close proximity in the same culture. (*B*) e2 (p11) cells after 7 d in culture form a large cyst; (*inset*) when grown in the presence of Trasylol for 14 d, e2 (p13) cells form narrow tubes (lumen indicated by *arrowheads*) instead of cysts. (*C*) u23 (p27) cells after 14 d in culture form a medium-sized cyst. (*D*) t38 (p22) cells after 6 d in culture form a tube-like structure; (*inset*) at higher magnification, a lumen (*arrowhead*) is clearly visible. (*E*) ut1 (p25) cells after 14 d in culture form a network of cell cords apparently devoid of a lumen; (*inset*) single isolated presumably nonproliferating ut1 (p27) cells are frequently observed in the same cultures. (*F*) p1 (p6) cells after 16 d in culture form a small cyst. Bar: (*A–F*) 125 μm; (*B*, *D*, and *E*, *insets*) 63 μm.

profile of mutant End. cells, RNA expression and zymographic analysis were performed. As shown in Fig. 3 *A*, both uPA-deficient cell lines (u23 and u24) expressed tPA at significant levels but no detectable uPA. The tPA-deficient End. cells t37 and t38 had high levels of uPA and no detectable tPA, whereas PAI-1 deficient cells expressed either uPA (p3) or uPA and tPA (p1) at high levels, but no PAI-1. As expected, the two End. cell lines lacking both

PAs only expressed PAI-1 (Fig. 3 *A*). Zymographic and reverse zymographic analysis (Fig. 3 *B* and Table II) confirmed the genotype of the cells. Thus, uPA, tPA, and PAI-1 activity was undetectable in cell lines derived from the corresponding knockout mice. Although we previously indicated that PAI-1 activity was undetectable by reverse zymography in two wild-type End. cell lines, namely b1 and e2 (Montesano et al., 1990), in the four additional wild-type cell lines assessed in this study, PAI-1 activity was detected (data not shown). Similarly, PAI-1 activity was detected in virtually all uPA−/−, tPA−/−, and utPA−/− End. cell lines (Table II).

Morphogenetic Behavior of Mutant End. Cells in Fibrin Gels

We have previously reported that End. cells form large cystlike structures lined by a monolayer of endothelial cells when embedded in three-dimensional fibrin gels in vitro (Montesano et al., 1990). In the present study we have observed that although all six wild-type cell lines have the capacity to form cysts in fibrin gels, there is heterogeneity with respect to the size of the cysts and the frequency with which they form. In addition to cysts, the presence of narrow or ectatic tubes was observed in four out of six lines (Fig. 4 and Table II). When grown in fibrin gels in the presence of Trasylol, a broad-spectrum serine protease inhibitor, cyst formation was inhibited and tube-like structures were observed in most wild-type End. cell lines (Fig. 4 and data not shown).

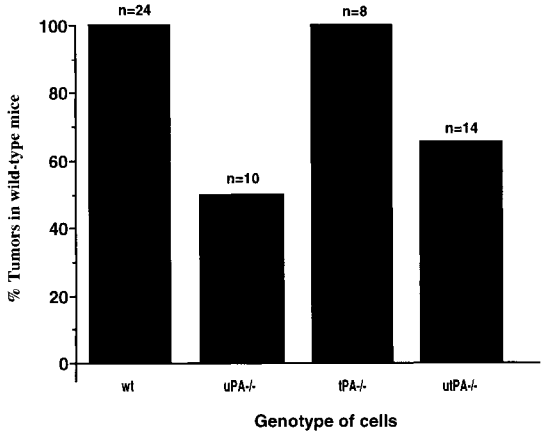
Both uPA−/− cell lines (u23 and u24) retained their capacity to form medium to small cysts (Fig. 4 and Table II). Cyst formation was completely inhibited in the presence of Trasylol, demonstrating the requirement for serine protease activity (data not shown). However, in contrast with wild-type cells, tube formation was never observed in the presence of Trasylol in either uPA−/− cell line; instead these cells either formed multicellular aggregates or remained as single isolated cells (data not shown). The two tPA−/− cell lines (t37 and t38) formed small cysts and occasional tube-like structures (Fig. 4 and Table II). Cyst and tube formation could be prevented by addition of Trasylol to t37 but not to t38 cells (data not shown). In striking contrast with most of the cell lines described thus far, both utPA−/− cell lines (ut1 and ut2) completely lost their capacity to form cysts (Fig. 4 and Table II). In addition, two clearly distinguishable cell populations could be identified: cells either organized into a network of cell cords or remained isolated as single rounded cells (Fig. 4 and Table II). Finally, PAI-1–deficient cells formed cysts, although in later passage p1 cells, rapid and massive fibrinolysis precluded the formation of either cysts or tubes (Fig. 4 and Table II). p3 cells appeared to be heterogeneous: in early passage cells, two cell populations that either formed cysts or cell cords could be identified. Cyst formation (in p1 and p3 cells) and fibrinolysis (in late passage p1 cells) could be inhibited by Trasylol. Cell cord formation by ut1, ut2, and p3 cells was unaffected by addition of Trasylol (data not shown). These findings show that, contrary to what might have been expected, uPA−/− and PAI-1−/− cells do not display a decreased or increased capacity to form cysts. The observation that the capacity to form cysts was reduced to a greater extent in tPA−/− cells than in uPA−/− cells suggests that, although both enzymes are capable of fibrinolysis in this system, tPA is more efficient.

Tumorigenic Potential of uPA-, tPA-, and utPA-deficient End. Cells in Adult Wild-Type Mice

To define a causal role for the PAs in End. cell–induced vascular tumors, we have used cells lacking uPA, tPA, and both uPA and tPA (utPA) for tumor formation studies in adult wild-type mice. We have used immunologically competent adult mice in these studies to avoid variability that may arise from the use of newborn mice with a developing immune system. Tumors were visible as small outgrowths in all mice injected with wild-type End. cells between day 4 and 7 and grew in size to 125 mm^2 by 11–18 d (Fig. 5 *A*). Histologically, these tumors consisted of a central hemorrhagic/necrotic core surrounded by an intense inflammatory cell infiltrate with neovascularization and very little fibrin deposition (Fig. 6, *A* and *B*). Similar results were obtained in mice injected with tPA-deficient End. cells, and there was no significant delay in either the onset or growth rates of these tumors. In contrast, tumor induction with uPA−/− End. cells showed incomplete penetrance and a delay in formation (Fig. 5 *A*). Only 50% of the mice developed tumors by days 11–14, and it took between 18 and 25 d for these tumors to reach maximum size. The remaining mice did not develop tumors for the entire 60-d observation period. Similarly, about two-thirds of the mice injected with End. cells deficient in both uPA and tPA developed tumors at an extremely slow rate (Fig. 5 *A*). Tumors were visible between 19 and 24 d after injection, and it took between 40 and 60 d to form tumors of maximum size. The remaining mice did not develop tumors. Histologically, these tumors were similar to those induced by wild-type cells, with the notable exception that extensive fibrin deposition was observed with the Lendrum and PTAH staining techniques (Fig. 6, *E* and *F*; data not shown). These results indicate that End. cell lines lacking only tPA activity are not inhibited in their capacity to form tumors. However, cell lines lacking uPA activity (both uPA−/− and utPA−/−) display a reduced efficiency in tumor formation in wild-type mice; when tumors do arise, their growth rate is significantly retarded, indicating that uPA activity of tumor cells is critical for efficient tumor growth.

To rule out the possibility that the observed differences in tumor formation rates were due to various degrees of histoincompatibility, we performed the tumor formation studies in immunoincompetent nude mice. After injection of wild-type and tPA−/−End. cells, tumors were visible around day 5 and grew in size to 125 mm^2 by 10–11 d (Fig. 5 *A*). In contrast, tumors were only visible between 7 and 14 d after injection with uPA−/− End. cells, and these tumors grew in size to 125 mm^2 by 18–22 d (Fig. 5 *A*). Similar results were obtained when utPA−/− End. cells were used, with tumors being visible between 10 and 14 d and reaching maximum size by 22–26 d (Fig. 5 *A*). These results in immunoincompetent mice confirm that the lack of tPA activity in End. cell lines does not affect their tumorigenic potential, whereas lack of uPA activity retards the

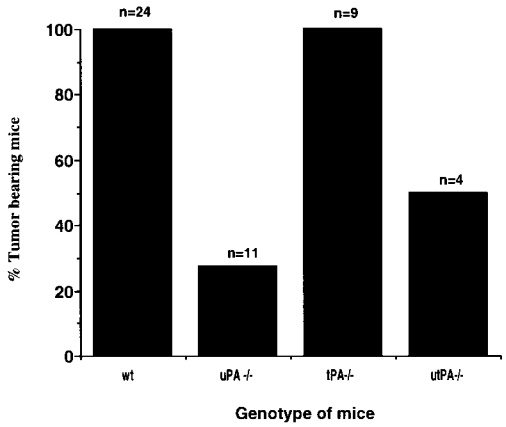
A **Vascular tumor formation using mutant End. cells in wild-type and nude mice**



Genotype of cells	Wild-type	uPA -/-	tPA -/-	utPA -/-
Onset of tumors in wild-type mice	4-7	11-14	4-7	19-24
Time taken to reach 125 mm^2 (days)	11-18	18-25	14-18	40-60
Onset of tumors in nude mice*	5-6	7-14	4-6	10-14
Time taken to reach 125 mm^2 (days)	10-11	18-22	10	22-26

*) 3 nude mice per group were injected with wild-type, uPA-/- and tPA-/- End. cells and 4 mice were injected with utPA-/- End. cells.

B **Vascular tumor formation using wild-type End. cells in mutant mice**



Genotype of mice	Wild-type	uPA -/-	tPA -/-	utPA -/-
Onset of tumors	4-7	12-14	4-7	18-20
Time taken to reach 125 mm^2 (days)	11-18	25-27	14-20	40-50

C **Vascular tumor formation using utPA -/- End. cells in mutant mice**

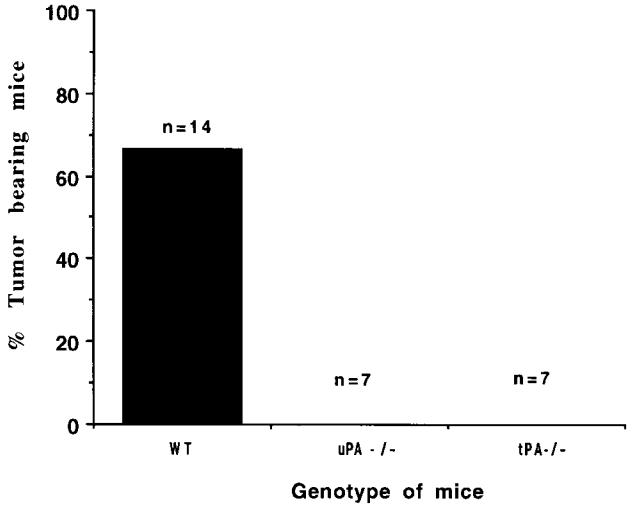


Figure 5. Incidence and latency of tumor formation. End. cell–induced tumor formation was investigated using mutant End. cells in wild-type and nude mice (*A*), wild-type End. cells in mutant mice (*B*), and utPA−/− End. cells in mutant mice (*C*). All mice were inbred for more than six generations and injected with the following cell lines; the time taken for the onset of tumors (days after injection) and the latency period for the formation of 125-mm^2 tumors was recorded: wild-type, s1 (p35 and 40) and s2 (p23, 34 and 40); uPA−/−, u23 (p33) and u24 (p23); tPA−/−, t37 (p25 and 35) and t38 (p6 and 25); and utPA−/−, ut1(p20) and ut2 (p28 and 40). Observation period was for up to 60 d after injection. *n*, number of mice from at least two independent experiments.

tumorigenic potential of both uPA−/− and utPA−/− End. cell lines.

Tumor Induction by Wild-Type and Mutant End. Cells in Adult uPA-, tPA-, and utPA-deficient Mice

We next investigated if adult mice lacking uPA, tPA, or both utPA are susceptible to tumor induction by wild-type End. cells. Cells were inoculated into the various mutant mice and tumor growth rates were scored (Fig. 5 *B*). No significant delay was observed in the onset of tumor development in tPA-deficient mice when compared with wild-type controls, although the time taken to reach maximum-sized tumors was slightly delayed (between 14 and 20 d in tPA-deficient mice as compared with 11–18 d in wild-type mice). However, tumor growth was delayed and variation

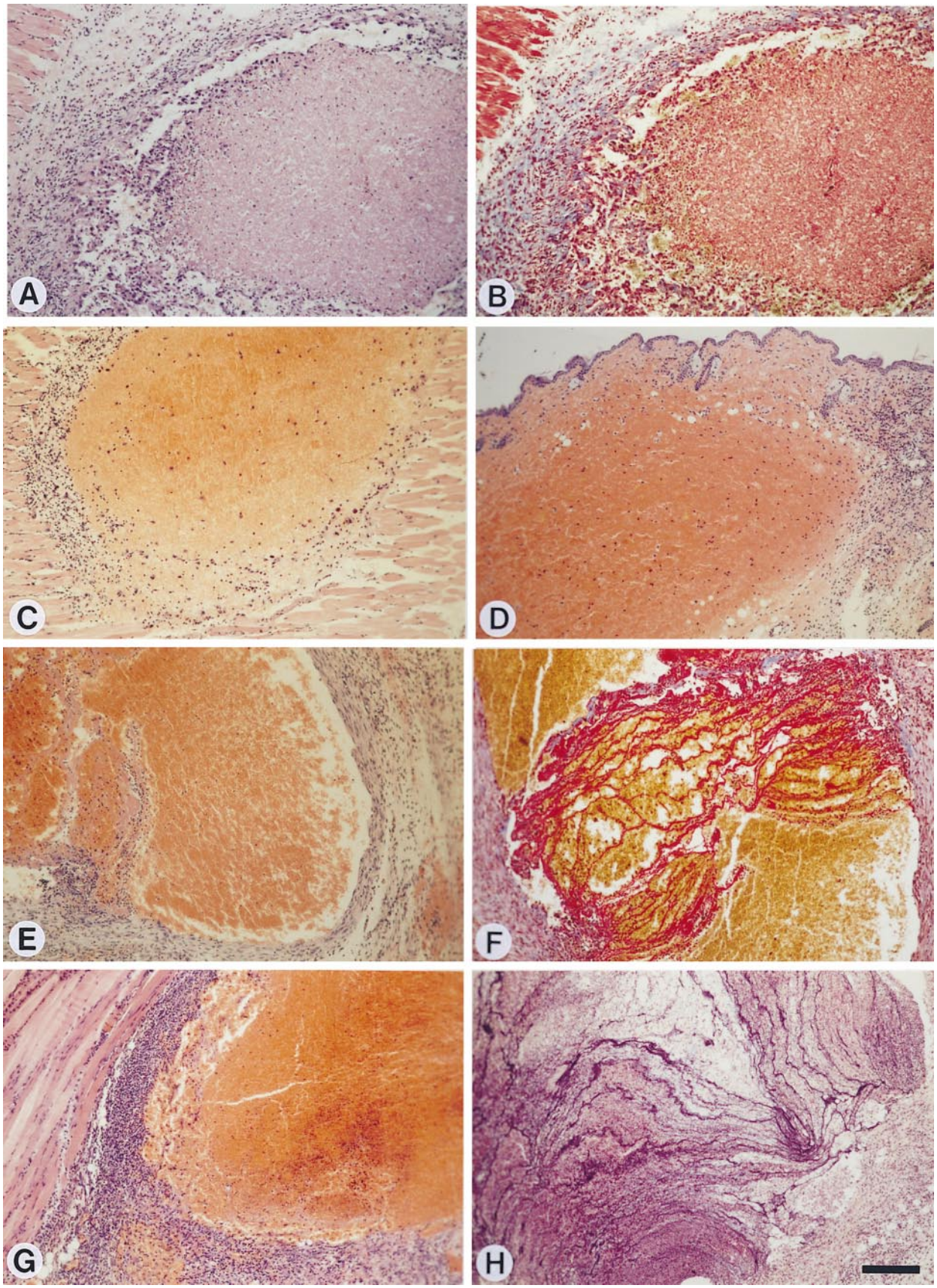


Figure 6. Morphology of End. cell–induced tumors in wild-type and mutant adult mice. (*A*) s1-induced subcutaneous tumor in a wild-type mouse after 3 d; *B* is the consecutive section stained with the Lendrum technique: note the absence of fibrin deposition, (*C*) b3-induced subcutaneous tumor in a tPA−/− mouse, and (*D*) s2-induced tumor in a uPA−/− mouse after 3 d. Note the presence of a cen-

in tumor formation rates was observed in uPA-deficient mice as follows: (*a*) only 3 out of 11 uPA-deficient mice developed tumors 12 to 14 d after injection, which grew to maximum size by day 27; (*b*) tumors were also visible in two other uPA-deficient mice between 12 and 14 d, but these tumors subsequently regressed; (*c*) the remaining six mice did not develop tumors throughout the entire 60-d observation period. Of the utPA-deficient mice injected with wild-type cells, only two out of four had visible tumors 18–20 d after injection, which then progressed to 125-mm^2 tumors by days 40–50. The remaining two mice did not develop tumors for the entire observation period. Only four utPA−/− mice could be used in this study since most of these mutant mice, even uninjected, became ill as they grew older (Carmeliet et al., 1994). Histologically, tumors induced by wild-type cells in mutant mice (Fig. 6, *C* and *D*) were similar to those induced in wild-type mice (Fig. 6, *A* and *B*), both at early (3 d; Fig. 6, *C* and *D*) and late (27 d; Fig. 6 *G*) time points. However, tumors induced by wild-type cells in utPA−/− mice were consistently associated with extensive fibrin deposition, as seen using the Lendrum and PTAH staining techniques (Fig. 6 *H* and data not shown). These results indicate that the host's PA status, and in particular the presence of uPA activity, plays a critical role in determining if proteolytically competent tumor cells can proliferate in vivo.

Since lack of uPA activity in both the host and the injected tumor cells affected the efficient formation of tumors, we next investigated the potential of utPA−/− End. cells to form tumors in mice lacking either uPA or tPA. Tumor formation by utPA−/− End. cells was inhibited in tPA−/− mice, since only three of the seven tPA−/− mice initially developed tumors between days 16 and 21; however, these tumors regressed by day 40 in all cases (Fig. 5 *C*). The remaining mice remained tumor free. utPA−/− End. cells did not form tumors in any of the uPA−/− mice during the entire observation period (Fig. 5 *C*). These results suggest that, although the lack of tPA alone has no significant effect on tumor formation, the uPA status of both the host and invading tumor cell has an important role in determining the progression of End. cell tumors.

Discussion

The specific formation of vascular tumors by PymT antigen provides an excellent system to study the molecular mechanisms underlying endothelial cell transformation. One of the components of this process is extracellular matrix degradation and invasion of surrounding tissues, which in the case of PymT results in a profound subversion of normal vasculature. In an attempt to analyze the role of the PA/plasmin system in PymT-mediated oncogenesis, we have investigated its ability to transform endothelial cells in mice lacking uPA, tPA, both uPA and tPA (utPA), or Plg. We found that the proteolytic status (PA/plasmin system) is not a critical determinant, and hence is not limiting for PymT-induced tumor formation. Moreover, the lack of either uPA or tPA activity does not affect the establishment of End. cells in vitro, although the combined loss of both PA activities leads to a reduced proliferation rate. Cyst formation by End. cells in fibrin gels in vitro could be correlated with altered proteolytic activity only in cells lacking uPA and tPA. However, End. cell–induced tumor formation in vivo, as opposed to tumors induced by PymT itself, is dependent on the proteolytic status (PA/plasmin system) of both the tumor cells and the host.

The finding that PymT was able to induce vascular tumors efficiently in the absence of uPA, tPA, utPA, and Plg indicates that the primary transformation process is independent of the PA/plasmin proteolytic status of the endothelial cells. This suggests that PymT-induced tumors can only be used as an in vivo assay for determining the role of this oncogene and associated signaling components in the initial transformation process. To date, the only signaling molecule thus identified is the Src-like kinase, Yes, since PymT could not efficiently transform endothelial cells in Yes-deficient mice in which the latency for tumor formation was also increased (Kiefer et al, 1994*b*).

Mutant End. cells lacking either uPA or tPA show no proliferation defect in culture. However, End. cell lines lacking both uPA and tPA require a longer time for establishment (three times longer compared with wild-type End. cells) and, when established, exhibit a marked reduction in cell proliferation in vitro. This proliferation defect is also exemplified in End. cell–induced tumor formation in vivo where cells lacking both PAs do not form tumors efficiently, and, when they do, the time taken to reach maximum size is about three times that seen with wild-type End. cells.

Assessment of the morphogenetic behavior of mutant End. cells in fibrin gels in vitro revealed that, although tPA appeared to be more efficient, the absence of either uPA or tPA alone did not render End. cells incapable of forming cysts, indicating that one PA is sufficient. However, combined loss of both uPA and tPA prevented cyst formation, clearly indicating that PA activity is required. The previous prediction that the "proteolytic balance," as represented by the ratio of PA:PAI-1, would predict the behavior of End. cells in fibrin gels (Montesano et al., 1990) therefore appears to hold true only when both PAs are absent. These findings indicate that the in vitro system only partially mimics the in vivo situation, and that the behavior of End. cells in fibrin gels is a poor predictor of End. cell tumorigenesis.

The incidence and rate of tumor formation in wild-type mice using End. cells lacking tPA activity were not signifi-

---

tral hemorrhagic/necrotic core surrounded by extensive peritumoral host cell recruitment (including inflammatory cells) and neovascularization in all sections (*A–D*) irrespective of cell or mouse genotype at this early time point (3 d). (*E*) ut1-induced tumor in a wild-type mouse after 40–45 d. (*F*) The consecutive section from the same tumor (which has been rotated slightly): staining with the Lendrum technique reveals extensive fibrin deposition, (G) s2-induced tumor in a uPA−/− mouse after 27 d, (*H*) s2-induced tumor in a utPA−/− mouse after 30 d; staining with the PTAH technique reveals extensive fibrin deposition. Note the persistence of peritumoral host cell recruitment in all sections (*E–H*) irrespective of cell or mouse genotype at this late time point (27–45 d). All sections stained with hematoxylin and eosin unless otherwise stated. Bar, 170 μm.

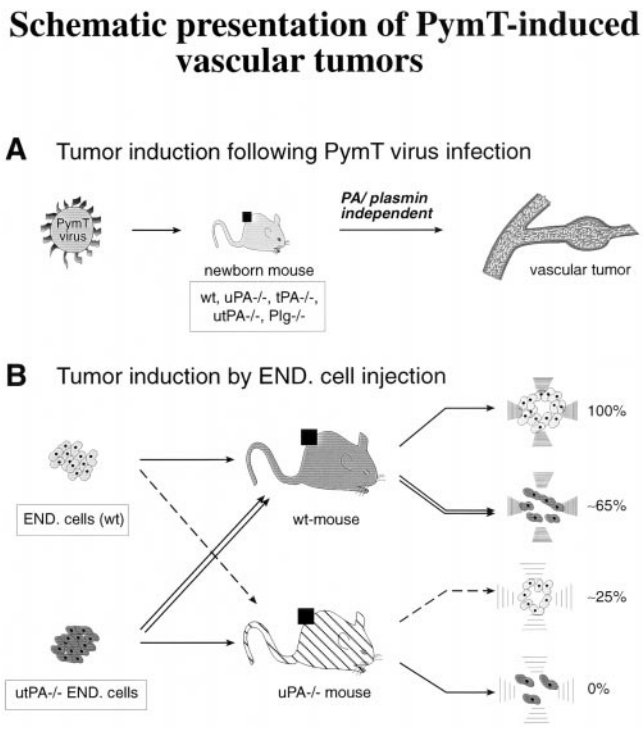


Figure 7. Proposed model depicting the formation of PymT-induced vascular tumors in newborn mice (*A*) and the formation of End. cell–induced vascular tumors in adult mice using mutant cell lines and mutant mice (*B*). The black box indicates the tumor. The arrows in *B* indicate the nature of the injected End. cells and the recipient mice, i.e., (*dashed arrows*) demonstrate that the transfer of wild-type End. cells into uPA−/− mice gives rise to vascular tumors in only 25% of the mice; in contrast, no tumors developed when utPA−/− End. cells were injected into uPA−/− mice (*solid arrow*s). The intensity of the lines in B (*right*) represents the extent of host cell interaction with the injected End. cells.

cantly affected. However, there was a marked decrease in the number of mice that developed tumors when injected with uPA−/− and utPA−/− End. cells. When tumors did form in a subset of these mice, the onset was delayed and the time taken to develop to maximum size was increased. In some cases, mice developed tumors initially, but these subsequently regressed completely. The data from tumor formation studies with nude mice ruled out the possibility that various degrees of histoincompatibility may have arisen during the establishment or the maintenance of mouse strains and tumor cell lines, thus affecting the experimental outcome. While wild-type and tPA−/− cell lines still formed tumors with 100% penetrance in nude mice, both uPA−/− and utPA−/− End. cell lines showed a delay in the onset of tumor formation as well as an increase in the time taken to form 125-mm^2 tumors. Furthermore, we have previously generated numerous End. cell lines lacking functional *c-src*, *fyn*, and *yes* genes in the same genetic background, C57Bl/6 × 129. These cell lines have been used in similar tumorigenicity studies, and no variations in the tumorigenic profile were found (Kiefer et al., 1994). Thus, the observed delay in End. cell–induced tumor formation and the consequent low incidence of tumor-bearing mice are not likely to result from genetic variations but could be from inefficient matrix degradation and a reduction in migratory/invasive properties of the tumor cells, which in turn lead to inefficient tumor expansion. This observation that End. cell–induced tumors cannot be efficiently formed when endogenous uPA is lacking but are independent of tPA may reflect the longstanding notion that uPA is required in situations of cell migration, morphogenesis, and tissue remodeling, while tPA is primarily implicated in intravascular thrombolysis.

The finding that mice lacking uPA activity (both uPA and utPA null mice) do not display efficient formation of wild-type End. cell–induced tumors was surprising. There was a marked decrease in the formation of tumors, and, when formed, there was a delay in the onset and latency. This indicates that the host's proteolytic status is also critical for the efficient formation of these tumors. Although the exact role of the host's uPA activity in tumor formation is presently unclear, it could be envisaged that the uPA proteolytic mechanism has to involve concerted interactions between both the invading tumor cells and the host's matrix for efficient fibrinolysis and subsequent matrix degradation. Thus, the lack of uPA activity in either would result in inefficient colonization by the tumor cells, as was shown here.

In conclusion, our studies have revealed that the pathogenesis of PymT-induced and End. cell–induced tumors is inherently different. In the former, the PymT virus infects endothelial cells in an existing vascular tree without the requirement for the PA/plasmin system in the initial transformation process (Fig. 7 *A*). Therefore, PymT-mediated vascular tumor formation appears to be PA/plasmin independent. On the other hand, in End. cell–induced tumors, the injected cells first have to invade host tissues and the basement membrane of existing blood vessels to gain access to the circulation to integrate within host vessels. This invasive process, which is reminiscent of tumor cell intravasation into the vascular tree, is dependent on both the host's and invading tumor cell's PA/plasmin status (Fig. 7 *B*). In addition, proliferation is impaired in cells entirely lacking PA activity. Defects in both invasion and proliferation, both of which appear to be PA dependent, are therefore likely responsible for the reduction in End. cell–induced tumor growth. Whether other proteases such as the MMPs, which have been shown to be involved in vascular tumor formation in vivo (Taraboletti et al., 1995), may play a role in this process has to be investigated in future experiments.

We thank Dr. V. Ploplis for the Plg−/− mice used in this study. We also thank Drs. H. Beug and M. Sibilia for critical reading of the manuscript, Drs. J.-D. Vassalli and S. Fisher for helpful comments, and Dr. L. Orci for continuing support. Technical assistance was provided by C. Di Sanza, M. Quayzin, and J. Stadler, and photographic work was done by B. Favri, G. Negro, and H. Tkadletz. This work was partly supported by the Austrian Industrial Research Promotion Fund and by grants from the Swiss National Science Foundation (31-34097.92 and 31-43364.95).

Received for publication 26 July 1996 and in revised form 28 February 1997.

References

Brizuela, L., L.M. Olcese, and S.A. Courtneidge. 1994. Transformation by middle T antigens. *Semin.Virol* . 5:381–389.

Bugge, T.H., M.J. Flick, C.C. Daugherty, and J.L. Degan. 1995*a*. Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. *Genes & Dev*. 9:794–807.

Bugge, T.H., T.T. Suh, M.J. Flick, C.C. Daugherty, J. Rømer, H. Solberg, V. Ellis, K. Danø, and J.L. Degan. 1995*b*. The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. *J. Biol. Chem*. 270:16886–16894.

Bugge, T.H., M.J. Flick, M.J.S. Danton, C.C. Daugherty, J. Rømer, K. Danø, P. Carmeliet, D. Collen, and J.L. Degan. 1996. Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. *Proc. Natl. Acad. Sci. USA*. 93:5899–5904.

Carmeliet, P., L. Kieckens, L. Schoonjans, B. Ream, A. Van Nuffelen, G. Prendergast, M. Cole, R. Bronson, D. Collen, and R.C. Mulligan. 1993*a*. Plasminogen activator inhibitor-1 gene-deficient mice 1. Generation by homologous recombination and characterization. *J. Clin. Invest.* 92:2746–2755.

Carmeliet, P., J.M. Stassen, L. Schoonjans, B. Ream, J.J. van den Oord, M. De Mol, R.C. Mulligan, and D. Collen. 1993*b*. Plasminogen activator inhibitor-1 gene-deficient mice 2. Effects on hemostasis, thrombosis, and thrombolysis. *J. Clin. Invest.* 92:2756–2760.

Carmeliet, P., L. Schoonjans, L. Kleckens, B. Ream, J. Degan, R. Bronson, R. de Vos, J.J. van den Oord, D. Collen, and R.C. Mulligan. 1994. Physiological consequences of loss of plasminogen activator gene function in mice. *Nature (Lond.)*. 368:419–424.

Danø, K., P.A. Andreasen, J. Grøndahl-Hansen, P. Kristensen, L.S. Nielsen, and L. Skriver. 1985. Plasminogen activators, tissue degradation, and cancer. *Adv. Cancer Res*. 44:139–266.

Dubois-Stringfellow, N., L. Kolpack-Martindale, V.L. Bautch, and R.G. Azizkhan. 1994*a*. Mice with hemangiomas induced by transgenic endothelial cells. A model for the Kasabach-Merritt syndrome. *Am. J. Pathol.* 144:796–806.

Dubois-Stringfellow, N., J. Jonczyk, and V.L. Bautch. 1994*b*. Perturbations in the fibrinolytic pathway abolish cyst formation but not capillary-like organization of cultured murine endothelial cells. *Blood.* 83:3206–3217.

Kiefer, F., S.A. Courtneidge, and E.F. Wagner. 1994*a*. Oncogenic properties of the middle T antigens of polyomavirus. *Adv. Cancer Res.* 64:125–157.

Kiefer, F., I. Anhauser, P. Soriano, A. Aguzzi, S.A. Courtneidge, and E.F. Wagner. 1994*b*. Endothelial cell transformation by the polyomavirus middle T antigen in mice lacking Src-related kinases. *Curr. Biol*. 4:100–109.

Lendrum, A.C., D.S. Fraser, W. Slidders, and R. Menderson. 1962. Studies on the character and staining of fibrin. *J. Clin. Pathol. (Lond.)*. 15:401–413.

Montesano, R., M.S. Pepper, U. Möhle-Steinlein, W. Risau, E.F. Wagner, and L. Orci. 1990. Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing middle T oncogene. *Cell.* 62:435–445.

Pepper, M.S., and R. Montesano. 1990. Proteolytic balance and capillary morphogenesis. *Cell Differ. Dev*. 32:319–328.

Pepper, M.S., R. Montesano, S. Mandriota, L. Orci, and J.-D. Vassalli. 1996. Angiogenesis: a paradigm for balanced extracellular proteolysis during cell migration and morphogenesis. *Enzyme Protein.* 49:138–162.

Pepper, M.S., F. Tacchini-Cottier, T.K. Sabapathy, R. Montesano, and E. Wagner. 1997. Endothelial cells transformed by polyoma virus middle T oncogene: a model for hemangiomas and other vascular tumors. *In* Tumour Angiogenesis. R. Bicknell, C.E. Lewis, and N. Ferrara, editors. Oxford University Press. In press.

Ploplis, V.A., P. Carmeliet, S. Vazirzadeh, I. Van Vlaenderen, L. Moons, E.F. Plow, and D. Collen. 1995. Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. *Circulation.* 92:2585–2593.

Taraboletti, G., A. Garofalo, D. Belotti, T. Drudis, P. Borsotti, E. Scanziani, P.D. Brown, and R. Giavazzi. 1995. Inhibition of angiogenesis and murine hemangioma growth by Batimastat, a synthetic inhibitor of matrix metalloproteinases. *J. Natl. Cancer Inst.* 87:293–298.

Vassalli, J.-D., J.M. Dayer, A. Wohlwend, and D. Belin. 1984. Concomitant secretion of prourokinase and of a plasminogen activator-specific inhibitor by cultured human monocytes-macrophages. *J. Exp. Med.* 159:1653–1668.

Vassalli, J.-D., A.-P. Sappino, and D. Belin. 1991. The plasminogen activator/plasmin system. *J. Clin. Invest.* 88:1067–1072.

Wagner, E.F., and W. Risau. 1994. Oncogenes in the study of endothelial cell growth and differentiation. *Semin. Cancer Biol*. 5:137–145.

Williams, R.L., S.A. Courtneidge, and E.F. Wagner. 1988. Embryonic lethalities and endothelial tumors in chimeric mice expressing polyoma virus middle T oncogene. *Cell.* 52:121–131.