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

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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., 1994a). 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., 1994a).

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...
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 trypsinic 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., α2-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−/−, uPA/tPA−/− (utPA−/−), PAI-1−/−, and Plg−/− mice have been described (Carmeliet et al., 1993a, 1994; Plplosi 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).

Materials and Methods

Mice

The generation of uPA−/−, tPA−/−, uPA/tPA−/− (utPA−/−), PAI-1−/−, and Plg−/− mice has been described (Carmeliet et al., 1993a, 1994; Plplosi et al., 1995). These findings implicated immunoglobulin/von Willebrand factor (vWF) (Dakopatts, Copenhagen, Denmark).

Viruses

with the N-TKmT virus were obtained from the virion produced 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 × 10⁶ neo²-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

Macroskopically visible tumors from mice injected with PymT viral particles were explanted, and End. cell lines were established as previously described (Kiefer et al., 1994b). 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 × 10³–1 × 10⁶ per 25-cm² 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 × 10⁶ cells, and then scored for tumor formation. The times taken for the onset of tumors and the latency period to form tumors of 125 mm² (maximum size) were recorded. All mice were killed when they had 125-mm² 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., 1994b). The probes were kindly provided by D. Belin (University of Geneva, Switzerland) (PAI-1), 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-
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 × 10⁴ 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., 1994b). 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 antivWF 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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Latency</th>
<th>Penetrance</th>
<th>Name</th>
<th>Abbr.</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>uPA−/−</td>
<td>10–13</td>
<td>9/10</td>
<td>mEnd.u23</td>
<td>u23</td>
<td>mesentry</td>
</tr>
<tr>
<td>tPA−/−</td>
<td>11–13</td>
<td>8/8</td>
<td>mEnd.t37</td>
<td>t37</td>
<td>mesentry</td>
</tr>
<tr>
<td>Plg−/−</td>
<td>8–12</td>
<td>9/9</td>
<td>liEnd.p1</td>
<td>p1</td>
<td>lung</td>
</tr>
</tbody>
</table>

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

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.
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<sup>2</sup>/<sup>2</sup> 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 activity of the cell lines, we performed a series of zymographic analyses. The data presented in Table II demonstrate that the absence of uPA and PAI-1 leads to a significant reduction in the proteolytic activity of the cell lines in vitro. The absence of tPA and utPA did not significantly affect the proteolytic activity of the cell lines.

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cell lines</th>
<th>uPA&lt;sup&gt;*&lt;/sup&gt;</th>
<th>tPA&lt;sup&gt;*&lt;/sup&gt;</th>
<th>PAI-1&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Behavior in fibrin gels&lt;sup&gt;†&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>b1, b3, e2, s2, m1, s1</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>Variably large to small cysts and tubes (n = 39)</td>
</tr>
<tr>
<td>uPA&lt;sup&gt;2/--&lt;/sup&gt;</td>
<td>u23, u24</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>Medium to small cysts and tubes (n = 18)</td>
</tr>
<tr>
<td>tPA&lt;sup&gt;2/--&lt;/sup&gt;</td>
<td>t37, t38</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Small cysts and tubes (n = 14)</td>
</tr>
<tr>
<td>utPA&lt;sup&gt;2/--&lt;/sup&gt;, tPA&lt;sup&gt;2/--&lt;/sup&gt;</td>
<td>ut1, ut2</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>No cysts and no tubes; isolated cells and network of cell cords (n = 6)</td>
</tr>
<tr>
<td>PAI-1&lt;sup&gt;2/--&lt;/sup&gt;</td>
<td>p1, p3</td>
<td>+++</td>
<td>++</td>
<td>–</td>
<td>Variably large to small cysts and network of cell cords (n = 14)</td>
</tr>
</tbody>
</table>

<sup>*</sup>Semiquantitative estimate determined by zymography (uPA, tPA) and reverse zymography (PAI-1).

<sup>†</sup>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.
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 mm2 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 mm2 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 mm2 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
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 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-mm² 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.](image-url)
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 uPA-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-mm² 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 un.injected, 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 (6) 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 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 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, 1994b).

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-
Schematic presentation of PymT-induced vascular tumors

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 arrows). The intensity of the lines in B (right) represents the extent of host cell interaction with the injected End. cells.

significantly 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-mm² 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. 7A). 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 invasion into the vascular tree, is dependent on both the host’s and invading tumor cell’s PA/plasmin status (Fig. 7B). 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. Studler, 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


