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 oncoproteins are known to rapidly transform target tissues without the need for additional genetic events; one such viral oncoprotein is the Polyoma middle T antigen (PymT). 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 trypsin 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 Daniø 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 has been described (Carmeliet et al., 1993a, 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).

**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; 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 × 10⁶ neo5-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**

Macrosopically 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 primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgrow contaminating fibroblasts. Within 5–10 passages, a homogeneously growing primary cultures, since they outgro
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 of 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, 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 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**

| Genotype | Latency | Penetrance | Name | Abbr. | Origin |
|----------|---------|------------|------|-------|--------|
| wt+/+    | 8–10    | 29/30      | bEnd.1 | b1   | brain  |
|          |         |            | eEnd.2 | e2   | yolk sac |
|          |         |            | mEnd.3 | m3   | mesentry |
|          |         |            | sEnd.1 | s1   | skin    |
|          |         |            | sEnd.2 | s2   | skin thorax |
| uPA−/−   | 10–13   | 9/10       | mEnd.u23 | u23 | mesentry |
|          |         |            | sEnd.u24 | u24 | skin thorax |
|          |         |            | pEnd.t37 | t37 | mesentry |
|          |         |            | tEnd.u38 | t38 | pancreas |
| tPA−/−   | 11–13   | 8/8        | mEnd.137 | t37 | mesentry |
|          |         |            | pEnd.138 | 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     | 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.
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 $\approx$10 passages (Fig. 2). The proliferation rate of ut1 and ut2 cell lines was $\approx$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 and morphogenetic behavior of End. cells lacking uPA, tPA, utPA, and PAI-1, we performed experiments as described in Materials and Methods. The Genotype and Cell lines are presented in Table II. The following cell lines were used: b1, b3, e2, s2, m1, s1, u23, p30, t37, p27, ut1, and p28. The table shows the proteolytic activity and morphogenetic behavior of End. cells in fibrin gels for 4–16 d in the absence of Trasylol; n = number of experiments. Variably large to small cysts and tubes ($n = 39$) Medium to small cysts and tubes ($n = 18$) Small cysts and tubes ($n = 14$) No cysts and no tubes; isolated cells and network of cell cords ($n = 6$) Variably large to small cysts and network of cell cords ($n = 14$)

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

$^+$, high activity; $\approx$, 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

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.
The observation that the capacity to form cysts was re-

duced 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 com-
petent 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 hemor-
rhagic/necrotic core surrounded by an intense inflam-
matory cell infiltrate with neovascularization and very little
fibrin deposition (Fig. 6, A and B). Similar results were ob-
tained 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 devel-
oped 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 observa-
tion period. Similarly, about two-thirds of the mice in-
jected with End. cells deficient in both uPA and tPA de-
tivated 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 maxi-
um 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 exten-
sive 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 tu-
mors 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 re-
results in immunoincompetent mice confirm that the lack of
tPA activity in End. cell lines does not affect their tumori-
genic potential, whereas lack of uPA activity retards the

**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 het-
erogeneity with respect to the size of the cysts and the fre-
cuency 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 ca-
pacity 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 pro-
tease 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 re-
mained as single isolated cells (data not shown). The two
tPA−/− cell lines (t37 and t38) formed small cysts and oc-
casional tube-like structures (Fig. 4 and Table II). Cyst
and tube formation could be prevented by addition of Tra-
sylol 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 ca-
pacity 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 re-
mained 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 pre-
cluded 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 re-

PAs only expressed PAI-1 (Fig. 3 A). Zymographic and
reverse zymographic analysis (Fig. 3 B and Table II) con-
formed 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).

The Journal of Cell Biology, Volume 137, 1997 958
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 days after injection, which grew to a maximum size by day 27; (b) tumors were also visible in two other uPA-deficient mice between 12 and 14 days, but these tumors subsequently regressed; (c) the remaining six mice did not develop tumors throughout the entire 60-day observation period. Of the utPA-deficient mice injected with wild-type cells, only two out of four had visible tumors 18–20 days 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 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 days; Fig. 6, C and D) and late (27 days; 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., 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-

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 days). (E) ut1-induced tumor in a wild-type mouse after 40–45 days. (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 days. (H) s2-induced tumor in a utPA−/− mouse after 30 days; 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 days). All sections stained with hematoxylin and eosin unless otherwise stated. Bar, 170 μm.
Schematic presentation of PymT-induced vascular tumors

A Tumor induction following PymT virus infection

B Tumor induction by END. cell injection

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.

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 invasion of 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. Studier, 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. Olecse, 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. 1995a. 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. Dano, and J.L. Degan. 1995b. 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. Pendersgast, M. Cole, R. Bronson, D. Collen, and R.C. Mulligan. 1995a. 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. 1993b. 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. Kieckens, 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. Andreassen, J. Grøndahl-Hansen, P. Kristensen, L.S. Nielsen, and L. Skriver. 1985. Plasminogen activators, tissue degradation, and cancer. Adv. Cancer Res. 44:139–206.

Dubois-Stringfellow, N., L. Kolpack-Martindale, V.L. Bautch, and R.G. Azizkhan. 1994a. Mice with hemangiomas induced by transgenic endothelial cells. A model for the Kasabach-Merritt syndrome. Am. J. Pathol. 144:796–808.

Dubois-Stringfellow, N., J. Jonczyk, and V.L. Bautch. 1994b. 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. 1994a. Oncogenic properties of the midddle 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. 1994b. 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. 22:319–328.

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., I.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.

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