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Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization

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Abstract
Acquisition of invasive/metastatic potential through protease expression is an essential event in tumor progression. High levels of components of the plasminogen activation system, including urokinase, but paradoxically also its inhibitor, plasminogen activator inhibitor 1 (PAI1), have been correlated with a poor prognosis for some cancers. We report here that deficient PAI1 expression in host mice prevented local invasion and tumor vascularization of transplanted malignant keratinocytes. When this PAI1 deficiency was circumvented by intravenous injection of a replication-defective adenoviral vector expressing human PAI1, invasion and associated angio-genesis were restored. This experimental evidence demonstrates that host-produced PAI is essential for cancer cell invasion and angiogenesis.

Tumor cell invasion and metastatic processes require the coordinated and temporal regulation of a series of adhesive, proteolytic and migratory events[1]. The plasminogen activator (PA)-plasmin proteolytic system has been implicated in these processes. Urokinase-type (uPA) and tissue-type (tPA) plasminogen activators are serine proteases that catalyze the conversion of inactive plasminogen into plasmin, a broadly acting enzyme able to degrade a variety of extracellular matrix proteins and to activate metalloproteinases and growth factors[2,3]. Plasminogen and uPA bind to their specific receptors directing plasmin activity to the migrating tumor cell surface. The activities of PA are directly controlled by specific inhibitors, the PA inhibitors 1 and 2 (PAI1 and PAI2) (ref. 4).

Many studies have focused on the role of uPA in cellular invasion and metastasis. Much of the data supporting the role of uPA in these events derives from *in vitro* and *in vivo* experiments demonstrating a correlation between uPA expression and cell invasion and metastasis as well as reduction of metastatic potential by using natural or synthetic serine protease inhibitors, neutralizing antibodies to uPA or antisense oligonucleotides[5,6].

PAI1 may also be directly involved in cancer progression. Both tumor cells and capillary endothelial cells express higher levels of PAI1 than other cell types[7-9]. Surprisingly, this inhibitor is necessary for optimal invasion of cultured lung cancer cells[10], and an increasing number of clinical studies have demonstrated that high PAI1 levels indicate a poor prognosis for the survival of patients suffering from a variety of cancers[11-13]. However, as PAI1 is an acute-phase reactant[14], it remains undetermined whether the increased PAI1 levels causally contribute to, or rather are the consequence of, the malignancy.

Various observations indicate that the PA system may provide both surface-associated protease activity and an adhesion mechanism for cells through interaction with vitronectin. Deng *et al.* suggested that the balance between cell adhesion and cell detachment is governed by PAI1 (ref. 15). The PAI1-mediated release of cells attached to vitronectin seems to occur independently of the ability of PAI1 to function as a protease inhibitor and results from its direct interaction with vitronectin rather than with uPA (ref. 15). These data indicate new roles for PAH in cancer progression and invasion[16].

To evaluate the biological relevance of PAI1 produced by host cells during cancer progression and invasion, we implanted malignant murine keratinocytes of the PDVA cell line[17] into PAI1-deficient ($PAI1^{-/-}$) (refs. 18,19) and wild-type (WT) mice. These cancer cells, which produce both PAs and PAI1, invaded the tissues of WT mice but not those of PAI1-deficient mice. Moreover, PAI1-deficient hosts failed to vascularize the implanted PDVA cells. In these PAI1-deficient mice, malignant cell invasion of host tissues and tumor vascularization were restored when systemic PAI1 expression was obtained by injecting a recombinant adenoviral vector bearing the human gene. These observations emphasize the essential role PAI1 has in the process of cancer invasion.

Malignant cell invasion is blocked in $PAI1^{-/-}$ mice

When PDVA keratinocytes were implanted onto the dorsal muscle fascia of WT mice (Fig. la), their growth pattern was typical of invasive malignant cells. As early as seven days after implantation, host-derived endothelial and stromal cells migrated upwards into the collagen gel (Fig. 1*b*). When the collagen gel was replaced by granulation tissue, malignant cell invasion proceeded downwards, so that after two weeks, the entire collagen gel was replaced by both malignant and host cells (Fig. 1c). By three weeks, the malignant keratinocytes had penetrated deep into the host mesenchyme and were intermingled with host stromal cells (Fig. 1*d*). We scored the invasion by measuring the maximal depth of penetration of individual tumoral sprouts and by calculating the average distance of invasion. After one week, no migration had occurred (less than 50 μm: score, -). Malignant cells had penetrated from 150 to 300 μm downwards after two weeks (score, ++), and from 300 to 1200 μm after three weeks (score, +++) (Fig. 1).

In contrast, when PDVA cells were implanted into $PAI1^{-/-}$mice, they initially proliferated to form a multilayered surface epithelium, but failed to invade the host tissue at any time after implantation (one, two or three weeks; score, -) (Fig. 1*e, f* and *g).* Although replacement of the collagen gel by newly formed granulation tissue occurred in a manner similar to that in WT mice, albeit later, the tumor cells did not become invasive and remained an irregular stratified surface epithelium. This was surprising, as the malignant PDVA epidermal cells were theoretically well equipped with the necessary proteolytic machinery. Indeed, as assessed by ELISA (ref. 20), cells grown *in vitro* as a monolayer secreted elevated amounts of uPA (7.2 ± 0.6 ng/mg of protein), tPA (34.5 ± 0.5 ng/mg of protein) and PAI1 (21.6 ± 3.0 ng/mg of protein) into their conditioned medium.

Tumor vascularization is impaired in $PAI1^{-/-}$ mice

Transplantation of the PDVA tumor cells into WT mice induced an angiogenic response in the host tissue starting from vessels of the dorsal muscle and subsequently extending far up into the collagen gel (Fig. *2a*). Between one and two weeks after transplantation, the vessels sprouted into the tumor epithelium, which had now started to invade into the newly formed granulation tissue (Fig. *2b*). Soon, the invading tumor columns were surrounded by a rich capillary network (Fig. *2c*). Similar angiogenic responses below the collagen gel were induced by PDVA cells in $PAI1^{-/-}$ mice (689 ± 91 capillaries/mm^2; n = 8) and in WT mice (632 ± 188 capillaries/mm^2; n = 11; P = not significant) (Fig. *2b*). However, in sharp contrast to those in WT mice, capillaries in $PAI1^{-/-}$ hosts failed to pierce through the remodeled collagen gel and remained confined to the area beneath it (Fig. *2d, e* and *f*). Concomitantly, tumor cell invasion in $PAI1^{-/-}$ hosts was completely blocked, indicating that vascularization of the tumor transplant was permissive, or even necessary, for tumor invasion.

PAI1 is expressed in host mesenchymal cells

In situ hybridization of tumor transplants in WT mice detected PAI1 mRNA expression in host mesenchymal cells, often concentrated in sites adjacent to the malignant keratinocytes, two weeks after grafting (Fig. 3a) and, even more intensely, three weeks after transplantation (Fig. 3c). A sense RNA probe did not show any specific hybridization (Fig. 3b). *In situ* hybridization and immunolabeling of serial sections showed that PAI1 mRNA was expressed in endothelial cells at the migrating front of neovessels (visualized by labeling of collagen type IV) (not shown). At the same time, PAI1 immunoreactivity was detected in the tumor stroma (mesenchymal cells and extracellular matrix) (Fig. *3e*), whereas the malignant cells expressed low levels of PAI1 mRNA and were only weakly immunoreactive for PAI1 (single labeling) (not shown). In contrast, $PAI1^{-/-}$ host tissues were negative for PAI1 mRNA, whereas tumor cells were labeled as in WT mice (Fig. *3d*). As expected, host tissue in $PAI1^{-/-}$ mice showed only background levels of PAI1 antibody reactivity (Fig. 3*f*).

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Fig. 1 a, *Schematic cross-section through an implant protected by the silicone chamber,* ***b-g****, Invasive behavior of malignant mouse keratinocytes (PDVA cells) one (b, e), two (c, f) or three (d, g) weeks after implantation. Malignant cells were transplanted into WT mice (b, c, d) or PAI1$^{-/-}$ mice (e, f, g). Histological sections were stained with hematoxylin and eosin. C, carcinoma cells; G, collagen gel; H, host connective tissue. Bars, 100 μm.*

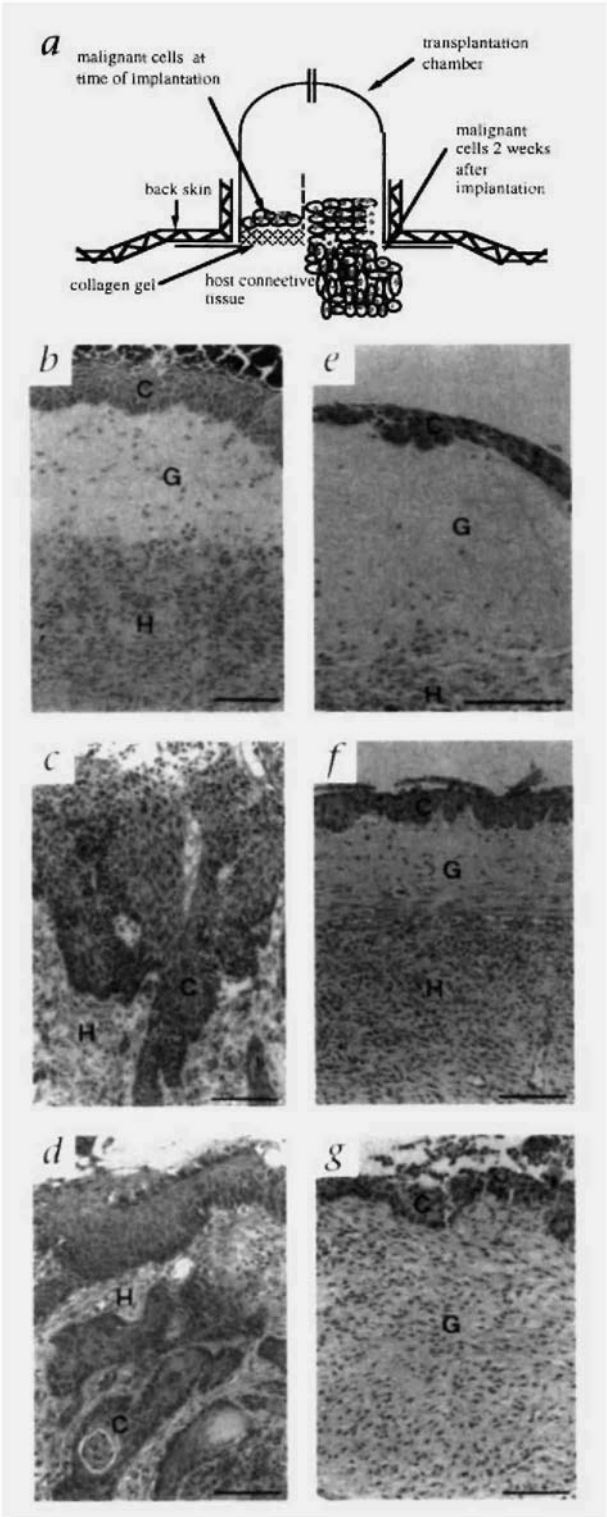

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Fig. 2 *Immunofluorescence labeling of malignant keratinocytes and vessels in transplants. PDVA cells implanted into WT mice (**a, b, c**) or PAI1$^{-/-}$ mice (**d, e, f**) and analyzed one (a, d), two (b, e) or three (c, f) weeks after implantation. Malignant cells were detected with anti-keratin antibody (in green) ; vessels, with anti-laminin (in red). At all times after grafting, laminin and collagen type IV labelings were always codistributed with endothelial cells recognized by the anti-mouse PECAM antibody (data not shown). C, carcinoma cells; C, collagen gel; H, host connective tissue. Bars, 100 µm.*

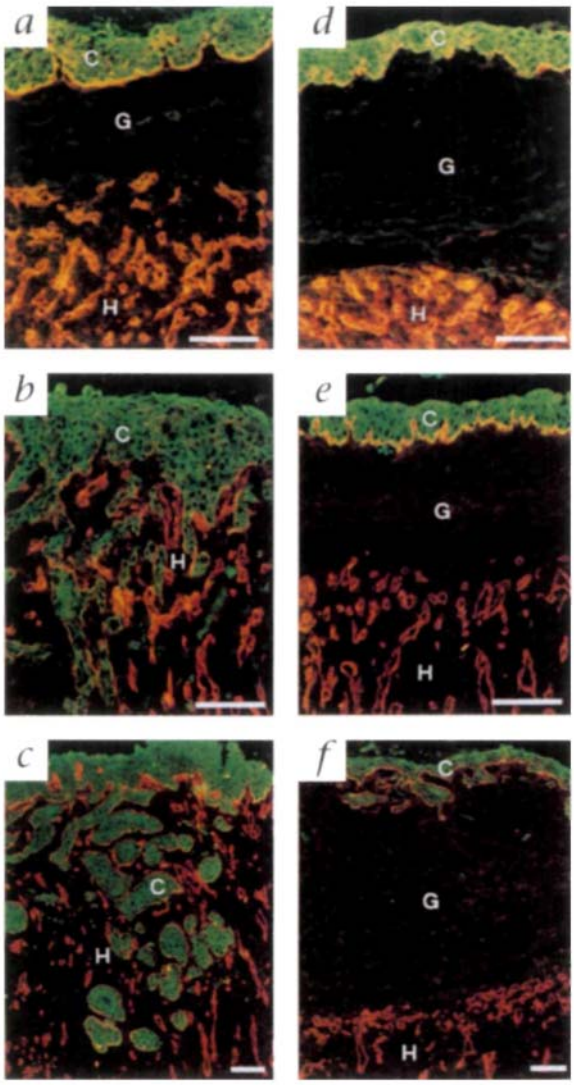

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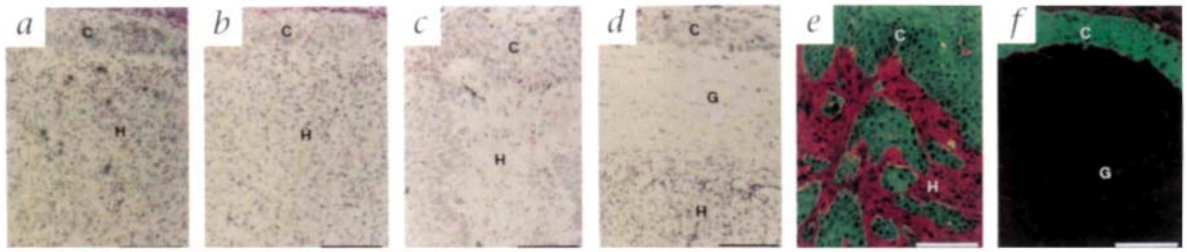
***Fig. 3 a-d**, In situ hybridization to PAI1 mRNA in malignant PDVA implanted into WT mice (a, b, c) or PAI1$^{-/-}$ mice (d) and analyzed two (a, b, d) or three (c) weeks after grafting. Adjacent sections were hybridized using mouse antisense PAI1 (a, c, d), and the corresponding mouse sense PAI1 (b) as a negative control. The sections were conterstained with hematoxylin and eosin and photographed under bright field microscopy, e and f, immunofluorescence labeling of PAI1 in WT mice (e) and PAI1$^{-/-}$ mice (f) two weeks after grafting. Rabbit polyclonal antibodies directed against PAI1 (e, f) were detected with a Texas red-conjugated secondary antibody. Guinea pig polyclonal antibody against keratin (malignant cells) was detected with FITC-conjugated secondary antibody (green) (e, f)· C, carcinoma cells; G, collagen gel; H, host connective tissue. Bars, 100 µm.*



Both genotypes have similar PA activity

PA activity was evaluated by *in situ* zymography which assesses the lysis of a casein mixture layered onto sections of three-week-old transplants (Fig. 4*a* and *b).* Within two hours, caseinolysis (seen as dark zones on dark-field images) was mostly confined to the invading front of tumor cells with strong angiogenesis in WT hosts (Fig. *Ac*). In PAI1$^{-/-}$ hosts, lysis was localized to the upper surface of malignant keratinocytes, which remained confined to the implant, and was found in the vascular-rich granulation tissue below the collagen gel (Fig. 4*d*)· This lysis was blocked by the uPA-specific inhibitor amiloride (2 mM) (Fig. 4*e* and *f),* therefore it was uPA-mediated. After six hours of incubation, caseinolysis became generalized over the entire sections of both WT and PAI1$^{-/-}$ transplants (Fig. *4g* and *h*). As demonstrated in the presence of amiloride, this reaction was also mostly uPA-mediated with only a minor fraction being tPA-mediated, and lysis was confined to the tumor cells in WT and the granulation tissue in PAI1$^{-/-}$ mice (Fig. 4 *i* and *j*). Thus, uPA activity was found chiefly in areas invaded by tumor cells and benefiting from angiogenesis, but overall no distinct differences between WT and PAI1$^{-/-}$ mice were found in the amounts of uPA or tPA-mediated lysis.

PAI1 adenovirus injection restores invasion and vascularization

To confirm the role of PAI1 in invasion and angiogenesis, on day one after implantation, PAI1$^{-/-}$ mice were injected with either a recombinant adenovirus (AdCMVPAI1) carrying human *PAI1* (n = 5) or with a control adenovirus (AdRR5) (n = 7), and grafted WT mice were injected with control adenovirus (AdRR5) (n = 5). As an additional control and to demonstrate the general distribution and infection by the virus, we intravenously injected AdCMV*LacZ* virus into PAI1$^{-/-}$ mice (n = 4). This injection resulted in the expression of LacZ preferentially in the liver but also next to the implant in areas where active angiogenesis had been found (Fig. 5a). LacZ expression could be localized to endothelial cells as assessed by double immunolabeling (data not shown). Four days after the injection of AdCMV*PAI1* the plasma levels of human PAI1 were higher than the normal murine PAI1 value in WT mice[19,21] (2 ng/ml) (Table). Four of five PAI1$^{-/-}$ mice injected with AdCMV*PAI1* had tumor invasion two weeks after implantation of PDVA cells (Fig. *5b).* This invasion pattern was similar to that seen in WT mice and the stroma of these tumors were also well vascularized (Fig. 5c). Injection with AdRR5 control virus did not influence the native behavior of malignant cells in either PAI1$^{-/-}$or WT mice (Table).

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Table: *Plasma levels of human PAI1 (hPAI1) in PAI1-/- mice on day four after injection of adenovirus*

Genotype	Virus	No. of mice	Plasma hPAI1 (ng/ml)	Invasion[a]
Wild-type	AdRR5	5	<0.8	+++
PAI1$^{-/-}$	AdRR5	7	<0.8	-
	AdCMVPAI1	5:		
		mouse 1	7	+
		mouse 2	10	-
		mouse 3	14	+
		mouse 4	29	++
		mouse 5	1,360	+++

[a]Score of average depth of invasion -: less than 50 μm; +: 50 < < 150 μm; ++: 150 < < 300 μm; +++: 300 < < 1200 μm.

Discussion

Recent reports indicate that high expression of PAI1 in tumors correlates with poor prognosis for patients suffering from gastric or breast cancer[22-24]. This apparent paradox indicated that the contribution of PAI1 to cancer progression and invasion is still poorly understood. Here, we investigated the role of PAI1 produced by host cells in the process of cancer cell invasion. The mouse skin carcinoma cells PDVA, cultured on collagen gel, were transplanted into PAI1-deficient mice or into WT mice. From these surface implants, the malignant keratinocytes invariably invaded the host stroma of WT mice, but invasion did not occur in PAI1$^{-/-}$ mice. PDVA cells produced and secreted elevated amounts of uPA, tPA and PAI1 in culture and had similar proteolytic activities whether implanted in WT or PAI1$^{-/-}$ hosts.

Thus, these data emphasize the key role in cancer invasion played by stromal cell-derived PAI1. Obviously, the PAI1 produced by the cancer cells was not sufficient to overcome the host cell deficiency. This conclusion was additionally strengthened by the finding that the invasive tumor phenotype in PAI1$^{-/-}$ mice was restored when these mice were injected with an adenovirus bearing human *PAI1*. The virus had infected stromal cells in tumor cell-induced granulation tissue and led to the detection of circulating human PAI1 at levels above normal murine PAI1 values in WT mice.

The absence of tumor cell invasion in PAI1-deficient mice could not be ascribed to a change in the production of uPA or tPA by the tumor cells, as lysis assessed by *in situ* zymography in the presence or absence of amiloride was similar in both WT and PAI1$^{-/-}$, nor could any increased proteolytic activity be seen in the PAI1$^{-/-}$ stroma.

Malignant keratinocyte transplants in WT, or PAI1$^{-/-}$ mice induced distinct angiogenic responses very similar to those elicited by human cancer cells in nude mice[25]. Indeed, in WT mice, neovessels rapidly formed within 1 week in the host tissue beneath the collagen gel. With other mesenchymal cells, these vessels sprouted into the collagen gel and soon after that were found to be in the immediate vicinity of the newly invading malignant cells. In contrast, capillaries in PAI1$^{-/-}$ mice remained beneath the collagen gel, even though other mesenchymal cells had colonized and replaced this matrix. Most surprisingly, tumor cells failed to invade this avascular granulation tissue. The composition of the matrix separating the tumor cells from the host stroma may have an important role in the different phenotypes observed. However, answer to this question will be addressed in future studies in which fibrin and matrigel or complex matrices will be used.

In situ hybridization and immunolabeling indicated that PAI1 was most strongly expressed at the advancing front of new blood vessels in WT mice. Furthermore, when PAI1 -deficient mice were injected with adenoviruses bearing human *PAI1,* high plasma concentrations of human PAI1 were measured, and cancer cell invasion and capillary sprouting into the tumor areas were restored. These findings strongly indicate that stroma-derived PAI1 is permissive and even essential for progression of angiogenesis, which in turn is essential for tumor invasion. This observation is in accordance with our recent finding that blockage of ongoing angiogenesis mediated by vascular endothelial growth factor receptor completely inhibited human tumor cell invasion when the same invasion model was used in nude mice[25]. Whether these important interactions between closely apposed blood vessels and tumor cells controlling malignant invasion are mediated by paracrine-acting growth and motility factors or by interactions with adhesion complexes needs additional investigation.

Although it has been suggested that PAI1 produced by tumor cells may confer protection to these cells[26,27], our results indicate that host-cell PAI1 has an essential role in regulating tumor vascularization and invasion. It has also been postulated that PAI1 produced by peritumoral stromal cells could represent a defense mechanism against extensive tissue destruction initiated by tumor cell proteolysis[26,27]. According to that theory, PAI1 gene deletion in host cells would then result in increased invasion and peristromal tissue destruction. Here we have provided evidence of the opposite effect and demonstrated that host PAI1 is essentially involved in tumor-associated neo-angiogenesis. In particular, in the absence of PAI1, capillaries failed to sprout into the vicinity of tumor cells and eventually into the tumor mass. Although endothelial cell migration and capillary sprouting have been proposed to require active proteolysis[28], it has been shown *in vitro* that excessive proteolysis prevents the coordinated assembly of endothelial cells into capillary shoots, indicating that proteolytic activity needs to be tightly controlled[29,30].

There is accumulating evidence that both the uPA receptor and PAI1 are multifunctional proteins involved not only in extracellular matrix proteolysis, but also in cellular adhesion and migration through their binding sites for vitronectin[16]. *In vitro* studies demonstrated that PAI1 blocks the binding between vitronectin and the uPA receptor expressed on the surface of endothelial cells[31], and interferes with vitronectin-integrin interactions[32]. Thus, PAI1 produced by tumor or stromal cells can impair cellular adhesion and promote or inhibit tumor cell detachment[33]. Whether PAI1 plays a regulatory role in angiogenesis by tightly controlling proteolytic activity or influencing cell migration on vitronectin remains to be determined. However, it seems obvious that the bioavailability of PAI1 to endothelial cells is essential for capillary sprouting in tumor angiogenesis and as a consequence, for tumor cell invasion.

Our findings might explain the apparently paradoxical clinical findings of a strong relationship between the levels of this protease inhibitor, high metastasis rate and poor survival[12-14].

Fig. *4 In situ zymography of PDVA cells three weeks after implantation into WT (σ, c, e, g, l) or PAI1$^{-/-}$ mice (b, d, f, h, i). PA activity was visualized as a dark zone of lysis (dark-field images) after incubation for two hours (c, d) or six hours (g, h). After of incubation in the presence of 2 mM amiloride for two hours (e, f) or six hours (i, j), only tPA activity was detected, a and b were stained with hematoxylin and eosin. C, carcinoma cells; G, collagen gel; H, host connective tissue. Bar, 1 mm.*

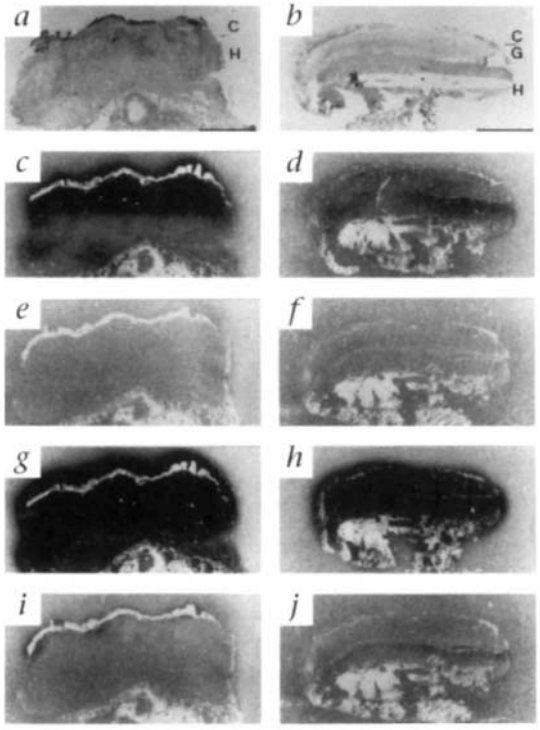

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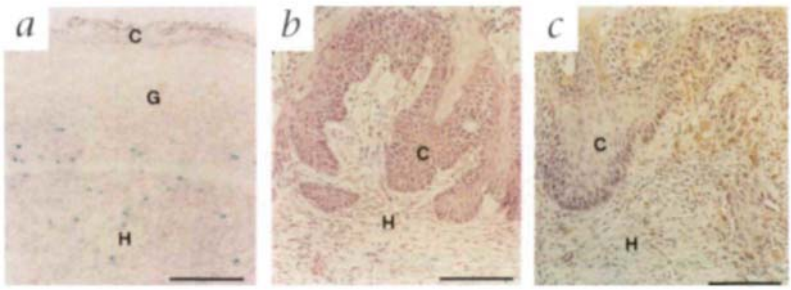
Fig. *5 a, After injection of PAI1 $^{-/-}$ mice with adenovirus bearing β-galactosidase (AdCMVLacZ), LacZ expression (in blue) was detected near the transplants, b, When PAI1$^{-/-}$ mice were injected with adenovirus bearing human PAI1(AdCMVPAI1), the tumor cells were able to invade the host mesenchyme, and showed the same behavior as that found in WT mice (Fig. 1 c). c, Immunohistochemical labeling with anti-collagen type IV antibody showed that the tumor was well vascularized (arrowheads). In all cases, tumor transplants were collected two weeks after grafting. C, carcinoma cells; G, collagen gel; H, host connective tissue. Bar, 200 μm.*



METHODS

Mice. Homozygous PAI1-deficient mice (PAI1$^{-/-}$) and the corresponding WT mice (PAI1$^{+/+}$) of either sex, with a mixed genetic background of 87% C57BL/6 and 13% 129 strain, were used throughout this study[18]. Croups were composed of 4-12 mice, as indicated.

Cell culture. Malignant murine keratinocytes (PDVA cells)(ref. 17) were routinely grown in modified Eagle's minimal essential medium containing a fourfold concentration of amino acids and vitamins (Life Technologies), 10% fetal calf serum (FCS) (Life Technologies) and antibiotics in a humidified incubator at 37°C, 5% CO_2. Cells (2×10^5) were plated on collagen gel (4 mg/ml of type I collagen isolated from rat tail tendons) inserted in Teflon rings (Renner, Dannstadt, Germany) and maintained in culture for one day before transplantation onto mice as described[34,35].

Transplantation assay in mice. Before transplantation, the cells were washed and the medium covering the cells was drained. The cell-coated collagen gels were then covered with a silicone transplantation chamber (Renner, Dannstadt, Germany) and implanted *in* toto onto the dorsal muscle fascia of mice as described[34,35] (Fig. 1 *a*). The mice were killed by cervical dislocation 7, 14 or 21 days later, and transplants were excised and either fixed *en bloc* for 24 h in buffered 3.5% formalin solution for routine histology or embedded in Tissue Tec (Miles Laboratories, Naperville, llinois) and frozen in liquid nitrogen for cryostat sectioning.

Because of the variable number of invasive sprouts and the different amounts of tumor mass in the different specimens, we measured the maximum depth of individual tumoral sprouts and calculated the average distance of penetration using the Quantimet 600 imaging[36] (Leica, Van Hopplynus, Brussels, Belgium).

Immunofluorescence. Cryostat sections (5 μm in thickness) were fixed in acetone at -20°C and in 80% methanol at 4 °C and then incubated with the primary antibodies. Antibodies raised against PECAM (rat monoclonal antibody, PharMingen, San Diego, California; diluted 1/20), 5'-bromo-2'-deoxyuridine (monoclonal antibody, Partec, Bottmingen, Switzerland; diluted 1/10), laminin (rabbit polyclonal antibody produced in our laboratory; diluted 1 /100), type IV collagen (guinea pig polyclonal antibody produced in our laboratory; diluted 1/100), keratin (guinea pig polyclonal antibody, Sigma; diluted 1/20) or β-galactosidase (rabbit polyclonal antibody, Europa Research Products, Cambridge, England; diluted 1/50) were incubated for 1 h at room temperature, whereas antibodies to PAI1 (rabbit polyclonal antibody produced in our laboratory, 10 μg/ml)[37] were incubated overnight at 4 °C. The sections were washed in phosphate buffered saline (PBS) (3x10 min) and then appropriate secondary antibodies conjugated to fluorescein-isothiocyanate (FITC), Texas red, or peroxidase were added: swine anti-rabbit (Dakopat, Glostrup, Denmark; diluted 1/40), rabbit anti-rat (Sigma; diluted 1/40), or mouse anti-guinea pig (Sigma; diluted 1/40) were applied for 30 min. For double immunofluorescence-labeling studies, sections were first incubated with the two primary antibodies, and then with FITC- and Texas red-conjugated secondary antibodies. After 3 washes in PBS for 10 min each and a final rinse in 10 mM Tris-HCI buffer, pH 8.8, coverslips were mounted and labeling was analyzed under an inverted microscope equipped with epifluorescence optics.

***In situ* hybridization.** A mouse *PAI1* cDNA fragment containing Pstl(338)-Xhol(857)[38] was subcloned into pBluescript KS(+) (Stratagene, La Jolla, California): pmPAI-1103 fragment. [^{35}S]UTP-labeled antisense or sense probes were generated by transcription of plasmids linearized BamHI or Xhol, using the relevant polymerases as described[39]. All probe preparations were adjusted to 1 x 10^6 cpm/ml.

In situ hybridization was done using the method described by Pyke *et al.* Paraffin sections were heated to 60 °C for 30 min, deparaffinized in xylene and rehydrated before treatment with proteinase K. After incubation overnight at 47°C with a hybridization solution containing the radiolabeled RNA probe, the sections were washed in 50% formamide, 10% dextran sulfate, tRNA (1 µg/µl), 0.02% Ficoll 400 (w/v), 0.02% polyvinylpyrrolidone (w/v), 0.2% bovine serum albumin fraction V (w/v), 10 mM DTT, 0.3 M NaCl, 0.5 mM EDTA, 10 mM Tris-HCl, and 10 mM Na_2HPO_4 (pH 6.8) twice for 1 h each wash. The sections were then treated with RNase A, dehydrated and air-dried. The slides were coated with autoradiographic emulsion and developed after 1 week of exposure at -20°C.

***In situ* zymography.** Cryostat sections were coated with a mixture containing 2% skim milk powder, 0.9% agar and 600 µg/ml of plasminogen (Sigma)(refs. 40,41). An 8% milk stock solution was prepared in PBS, heated at 95 °C for 30 min and briefly centrifuged at 1,000 *g.* to remove insoluble material. Slides were incubated at 37°C in a humidified chamber for 1 to 12 h. Caseinolysis in the coating mixture in the presence or absence of 2 mM amiloride (Sigma) was monitored by examination under a dark-field microscope.

Adenovirus-mediated *PAI1* gene transfer. Recombinant adenovirus vectors bearing human *PAI1* (AdCMVPAI1), *Escherichia coli* β-galactosidase *(AdCMVLacZ)* and control adenovirus (AdRR5) were generated as described[42]. One day after cell transplantation, mice were intravenously injected with 200 µl of control or recombinant adenovirus (7 x 10^8 PFU). After five days, blood was sampled from the retroorbital sinus and PAI1 antigen was measured as reported[42]. On day 14, mice were killed and transplants were excised and processed as described above.

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