Overexpression of Plasminogen Activator Inhibitor Type 2 in Basal Keratinocytes Enhances Papilloma Formation in Transgenic Mice

Hong-Ming Zhou, Isabelle Bolon, Anthony Nichols, Annelise Wohlwend, and Jean-Dominique Vassalli

Department of Morphology, University of Geneva Medical School, CH-1211 Geneva 4, Switzerland

ABSTRACT

The serpin plasminogen activator inhibitor (PAI) type 2 is expressed in differentiated epidermal keratinocytes. To explore its role in this tissue, we studied the impact of PAI-2 overexpression on epidermal differentiation and skin carcinogenesis. A mouse PAI-2-encoding transgene was targeted to basal epidermis and hair follicles under the control of the bovine keratin type 5 gene promoter. Two mouse lines were established, one of which strongly expressed the transgene and produced elevated levels of PAI-2 in the epidermis. Although it had no manifest impact on cellularity or differentiation of skin or hair follicles, PAI-2 overexpression rendered the mice highly susceptible to skin carcinogenesis induced by a single application of 7,12-dimethylbenz(a)anthracene (initiation) followed by twice weekly applications of 12-O-tetradecanoylphorbol-13-acetate (TPA (promotion)). In transgenic mice, papillomas could be observed after 3 weeks of promotion; after 8 weeks, 94% (31 of 33) of transgenic mice had developed readily visible papillomas, whereas only 35% (7 of 20) of control mice (transgene-negative littersmates) had barely detectable lesions. After 11 weeks, all but 1 (32 of 33) of the transgenic mice had papillomas as compared with only 65% (13 of 20) of control mice. After 11 weeks of promotion, application of TPA was terminated. In control mice, papillomas regressed and eventually disappeared; in transgenic mice, there was continued growth of papillomas, some of which further progressed to carcinomas. In contrast to massive apoptosis in regressing papillomas of control mice, only a few apoptotic cells were detected in transgenic papillomas after the cessation of TPA application. The effect of PAI-2 on papilloma formation did not appear to involve inhibition of the secreted protease urokinase-type plasminogen activator (upPA); PAI-2 accumulated predominantly in cells, and PAI-2 overexpression failed to alleviate a phenotype induced by uPA secretion, as demonstrated by a double transgenic strategy. In addition, in situ hybridization revealed that upPA mRNA is not expressed concomitantly with PAI-2 in developing papillomas. We conclude that overexpression of PAI-2 promotes the development and progression of epidermal papillomas in a manner that does not involve inhibition of its extracellular target protease, uPA, but appears to be related to an inhibition of apoptosis.

INTRODUCTION

PAI-2 are serpin-class antiproteases that inhibit uPA and tPA, two extracellular serine proteases. By converting plasminogen to plasmin, both uPA and tPA catalyze extracellular proteolysis and thereby play important roles in biological and pathological processes (1). This proteolytic cascade is controlled at different levels, one of which is inhibition of plasminogen activator catalytic activity by PAIs. Two different PAIs (PAI-1 and PAI-2) have been identified in mammals. PAI-1 is expressed more broadly than PAI-2, and its role in modulating extracellular proteolysis has been demonstrated by ablation (2) or overexpression (3) of the PAI-1 gene. Although PAI-2 can inhibit extracellular uPA and tPA (the two-chain form; Ref. 4), it may have additional intracellular functions: it is found in both a secreted and an intracellular cytosolic form, both of which result from translation of the same mRNA; and their antiprotease activity is similar (5–7). Hints regarding the possible functions of intracellular PAI-2 have come from the observations that induction of endogenous PAI-2 or addition of exogenous PAI-2 protects cells from Mycobacterium-induced apoptosis (8) and that cells transfected with PAI-2-encoding vectors are resistant to apoptosis (9) or cytolysis (10) or are protected against the rapid cytopathic effects of viral infection (11). These observations have led to the hypothesis that PAI-2 may exert intracellular functions involved in regulation of cell apoptosis and necrosis.

PAI-2 is expressed in a limited number of tissues and cells, including placenta (12), monocytes/macrophages (5), and epidermis (13–15). The epidermis is an interesting model system in which to study the role of PAI-2 in vivo. It comprises stem cells and transiently proliferating cells in the basal layer and differentiating cells in the suprabasal layers (16, 17). Epidermal differentiation culminates in the production of dead, flattened, enucleated squames consisting of keratin filaments surrounded by a proteinaceous, cross-linked cornified envelope and is thus a physiological process of apoptosis (18). PAI-2 is preferentially synthesized in differentiated epidermal cells in vivo and in vitro (15, 19) and can serve as a differentiation marker for keratinocytes. To investigate whether dysregulation of PAI-2 expression may disturb epidermal differentiation, we targeted overexpression of mouse PAI-2 to the proliferating population of mouse epidermis and hair follicle cells by placing a PAI-2-encoding transgene under the control of the K5 promoter (20). Transgenic mice were highly susceptible to chemically induced papilloma formation. Furthermore, PAI-2-overexpressing papillomas did not undergo extensive apoptosis on cessation of tumor promotion and therefore continued to develop.

MATERIALS AND METHODS

Generation of K5-PAI-2 Transgenic Mice. A cDNA encoding full-length mouse PAI-2 (a gift from Dominique Belin, Department of Pathology, University of Geneva Medical School) was cloned in the Xbal/BamHI sites of the pBSKS-II vector (Stratagen). From this, a Xbal/ClaI fragment was cloned downstream of the intron of the rabbit β-globin gene, in Xbal/ClaI sites that had been added through a double-stranded oligonucleotide adapter in the EcoRI site of the genomic sequence of the rabbit β-globin gene. Thus, the β-globin-PAI-2 construct contains an intron, the coding sequence of PAI-2, and the polyadenylation signal of rabbit β-globin. The K5 promoter was added by inserting a 5200-bp KpnI blunted/NolI fragment (20) in the SacII blunt/NolI site of β-globin-PAI-2 vector to generate the K5-PAI-2 construct. A 7910-bp fragment containing the K5 promoter, rabbit β-globin intron, full-length mouse PAI-2 cDNA, and polyadenylation signal (Fig. 1 A) was released with KpnI and SacII from the K5-PAI-2 plasmid, purified, and used to produce transgenic mice by pronuclear injection of fertilized CBA/B6 F1 embryos. PCR. This was conducted in a 50-μl mixture containing 1× PCR buffer, 200 μM deoxynucleotide triphosphates, 0.5 μM of primers designated GLO (5′-GCATATAAATTCT GGCTGGCGTGG-3′) and PAI-2 (5′-CTGGGTTCCTTGTGGTGATACC-3′), 1 unit of Taq DNA polymerase, and 1 μg of...
Carcinogenesis in Mice Overexpressing PAI-2

Fig. 1. The K5-PAI-2 transgene and identification of transgenic mice. A, K5-PAI-2 construct. From left to right, the transgene consists of the K5 promoter (black), a rabbit β-globin intron (white), the full-length mouse PAI-2 cDNA (gray), and the polyadenylation signal of rabbit β-globin (white). Sal I and Kpn I are the sites used to excise the construct from the plasmid. The sense primer GLO, starting at -261 and the antisense primer PAI2, starting at 233) used for PCR identification of transgenic mice are indicated. Within the amplified fragment, there is a unique Apal site at position -119. B, identification of K5-PAI-2 transgenic mice. The PCR products from wild-type (WT), founder 52, and founder 23 mice were resolved either directly or after Apal digestion in a 2% agarose gel and stained with ethidium bromide. The size of the whole fragment and the size of the two fragments from Apal digestion are indicated. As a marker (M), a 1-kb DNA ladder was run in parallel. C, in situ hybridization. A cryosection of transgenic (line 23) mouse skin was probed with digoxigenin-labeled antisense PAI-2 cRNA, and the hybridized probe was detected by alkaline phosphatase activity conjugated to antidigoxigenin antibody. PAI-2 mRNA is localized in basally located cells of the epidermis and the outer root sheath of hair follicles (arrows). D, PAI-2 assay. 35S-radiolabeled human uPA was incubated alone (uPA) or with skin extracts from a transgene-negative littermate (WT), a line 52, or a line 23 mouse. Samples were resolved by 10% SDS-PAGE, and the gel was processed for autoradiography. The presence of PAI-2 is revealed by the formation of a SDS-resistant uPA/PAI-2 complex.

RESULTS

Characterization of K5-PAI-2 Transgenic Mice. Transgenic mice were identified by PCR. A transgene fragment of the expected size was amplified from DNA samples of candidate founders 23 and 52 (Fig. 1B); the nature of the fragment was confirmed by digestion at a unique Apal site (Fig. 1A), which yielded two fragments of the expected sizes (Fig. 1B). Both individuals transmitted the transgene in a Mendelian fashion and were used as founders to establish lines 23 and 52. Transgenic mice were indistinguishable from their negative

Carbogenic DNA under the following conditions: 3 min at 95°C followed by 35 cycles at 94°C, 66°C, and 72°C (50 s/step). Aliquots (10 μl) of PCR reactions were resolved in 1% agarose gels and visualized by ethidium bromide staining. When identified, the positive PCR preparations were precipitated and digested with Apal; the digestion products were resolved and visualized as described above.

Generation of K5-PAI-2/K5-uPA Double Transgenic Mice. The generation and characterization of the K5-uPA transgenic line have been described previously (21). Phenotypically, K5-uPA mice are readily recognizable for having chalky white teeth because of a defect in enamel formation due to the enzymatic activity of transgene-encoded uPA, which is expressed in ameloblasts (21). Double transgenic mice were generated by crossing K5-PAI-2 with K5-uPA mice and identified by PCR using primers for K5-PAI-2 as described above and primers for K5-uPA (21). After having been genotyped, mice were anesthetized, and their incisors were photographed.

In Situ Hybridization. A PstI-EcoRI fragment of mouse PAI-2 cDNA was cloned into pBSKS. Constructs containing sense (SP65-muk) or antisense (SP64-muk) mouse uPA cDNA (PstI-HindIII) were kindly provided by Dominique Belin. The plasmids were linearized and transcribed with the appropriate enzymes to generate digoxigenin-labeled sense or antisense RNA probes, using a digoxigenin labeling kit (Boehringer), 0.2 μM digoxigenin-11-dUTP (Boehringer), and 1× reaction buffer at 37°C for 60 min. Incorporated digoxigenin-11-dUTP was revealed by an antidigoxigenin antibody conjugated to alkaline phosphatase (Boehringer) with 5-bromo-4-chloro-3-indoly-phosphate and nitroblue tetrazolium chloride as substrates. Results were recorded using a Zeiss microscope.

In Situ Detection of DNA Fragmentation. Papillomas were collected 3 weeks after TPA termination, fixed in 4% paraformaldehyde, and processed to yield 5-μm paraffin sections. The sections were dewaxed in xylene, rehydrated in graded ethanol solutions, and denatured in water at 70°C for 60 min. Tailing reactions were carried out in a mixture containing 1 μM digoxigenin-11-dUTP (Boehringer), 0.2 μM dATP, 0.5 unit/μl terminal transferase (Pharmacia), and 1× reaction buffer at 37°C for 60 min. Incorporated digoxigenin-11-dUTP was revealed by an antidigoxigenin antibody conjugated to alkaline phosphatase (Boehringer) with 5-bromo-4-chloro-3-indoly-phosphate and nitroblue tetrazolium chloride as substrates. Results were recorded using a Zeiss microscope.

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littermates in terms of body size, life span, coat fur, and fertility throughout a 2-year period of observation (data not shown). In situ hybridization performed on a sample from line 23 revealed PAI-2 mRNA in basally located cells of the epidermis and the outer root sheath of hair follicles (Fig. 1C); the specificity of the signal was confirmed using a corresponding sense probe, which did not hybridize to these sites (data not shown). No signal was detected in wild-type skin analyzed in parallel (data not shown); the signal observed in transgenic epidermis thus did not correspond to endogenous PAI-2 mRNA, which was under detection in our experiment. The presence of the transgene-encoded protein was also determined. Total protein extracts from an equal surface of skin from control, line 23, and line 52 mice were incubated with radiolabeled human uPA, resolved by SDS-PAGE, and autoradiographed. A uPA-PAI-2 complex of similar electrophoretic mobility was revealed in both the wild-type and the two transgenic lines (Fig. 1D), indicating that transgene-encoded PAI-2 was qualitatively similar to endogenous PAI-2. PAI-2 levels in wild-type and line 52 skin extracts were similar, whereas the amount of PAI-2 in line 23 mice was much higher (Fig. 1D). Thus, line 23 was selected for additional experiments, and, unless otherwise indicated, the term “transgenic mice” used hereafter refers to this line.

PAI-2 Expression in Basal Keratinocytes Does Not Alter Epidermal Differentiation. To investigate the impact of PAI-2 overexpression on epidermal structure, we compared the histology of adult back skin from wild-type and transgenic mice (Fig. 2). Consistent with the normal gross appearance of the skin, the two samples were indistinguishable in terms of epidermal thickness and hair follicle density and structure.

PAI-2 Overexpression Enhances Papilloma Formation. Papillomas were induced using the initiation (DMBA)-promotion (TPA) protocol. In transgenic mice, visible papillomas were observed after 3 weeks of promotion (data not shown). After 8 weeks, nearly all (31 of 33) transgenic individuals had readily recognizable papillomas (Fig. 3B), whereas only 7 of 20 control mice bore barely recognizable lesions (Fig. 3A). At the end of TPA application (week 11), all but 1 transgenic mouse (32 of 33) and 13 of 20 control mice had developed papillomas (97% versus 65%; P = 0.0016; Fig. 3C). Papilloma density (number of papillomas/papilloma-bearing mouse) was not significantly different between the transgenic (12.03 ± 6.60) and control (14.17 ± 7.58) groups. However, on average, papillomas in transgenic mice were of larger size, probably because they developed earlier than papillomas in control mice. In agreement with the low level of transgenic PAI-2 expression, carcinogenesis in line 52 mice was similar to that in control mice (data not shown). Treatment with DMBA or TPA alone did not result in any detectable lesions in transgenic or control mice, indicating that carcinogenesis in these
mice follows the multistep model (24) and that PAI-2 overexpression does not circumvent the requirement for either initiation or promotion.

**PAI-2 Overexpression Prevents the Regression of Papillomas.** After cessation of treatment with TPA, the papillomas in control mice progressively regressed and eventually disappeared (Fig. 4A); in transgenic mice, in contrast, they kept growing (Fig. 4B). Given that transgenic PAI-2 expression persists at the papilloma stage (Fig. 6), the differential response to TPA withdrawal suggests that PAI-2 prevents papilloma regression. Regression may involve apoptotic cell death. To verify this hypothesis, eight papillomas of comparable sizes were collected from four transgenic and four control mice 3 weeks after cessation of TPA treatment. Transgenic and control papillomas were analyzed in parallel for the presence of apoptotic cells by *in situ* DNA nick labeling. Comparable results were obtained for all transgenic and for all control samples, and a representative experiment from one pair of animals is shown in Fig. 4, C–F. Whereas in control papillomas, many cells were undergoing apoptosis (Fig. 4, C and E), very few cells were labeled in transgenic papillomas (Fig. 4, D and F). Thus, PAI-2 overexpression may prevent the regression of papillomas by decreasing apoptosis.

**Transgene-encoded PAI-2 Does Not Act by Inhibiting uPA.** Extracellular proteolysis has been implicated in keratinocyte apoptosis (25). Because PAI-2 can inhibit uPA, if it were secreted by keratinocytes, it might prevent apoptosis as a result of inhibiting uPA. To investigate this possibility, we first analyzed the distribution of PAI-2 protein in skin explant cultures (see Fig. 1D). PAI-2 was detected in the tissue extract but not in the conditioned medium (data not shown), suggesting that transgene-encoded PAI-2 as well as endogenous PAI-2 remains predominantly intracellular in keratinocytes.

Because such an *in vitro* assay may not faithfully represent the situation *in vivo*, we designed an *in vivo* strategy to evaluate the capacity of transgene-encoded PAI-2 to prevent uPA-mediated effects. This strategy is based on the finding that transgenic mice expressing uPA in the enamel epithelium, under the control of the K5 promoter, have abnormal tooth development and are easily recognized by their chalky white teeth (21). Expression of PAI-2 was also targeted to the enamel epithelium in K5-PAI-2 mice, as revealed by *in situ* hybridization (data not shown). Thus, in K5-uPA/PAI-2 double transgenic mice, if PAI-2 is secreted as is uPA, it should inhibit uPA activity and hence prevent the occurrence of the tooth phenotype. However, like K5-uPA mice, double transgenic mice had chalky white teeth (Fig. 5A), indicating that uPA activity had not been inhibited by PAI-2. The failure of PAI-2 to prevent the tooth phenotype could be due to insufficient PAI-2 expression. To evaluate this possibility, we compared the expression levels of transgene-encoded PAI-2 and uPA by measuring the net uPA activity of skin extracts from wild-type, K5-uPA, and K5-uPA/PAI-2 mice (Fig. 5B). Coexpression of PAI-2 in double transgenic mice reduced uPA activity to a level even lower than that in wild-type mice, indicating that transgenic PAI-2 expression was sufficient to abolish transgenic uPA activity. Taken together, these results suggest that in PAI-2 transgenic papillomas, the repression of apoptosis appears to be independent of inhibition of uPA but rather involves an intracellular effect of PAI-2.

**uPA and PAI-2 Are Not Concomitantly Expressed in Transgenic Papillomas.** To further explore the possibility that the effect of transgene-encoded PAI-2 on the formation and fate of papillomas may be mediated through inhibition of uPA, we compared uPA and PAI-2 expression in five growing transgenic papillomas by *in situ* hybridization (Fig. 6). Whereas transgenic PAI-2 was widely expressed in the basal layer of both normal skin and papillomas (Fig. 6A), very few cells expressed uPA at discrete sites (Fig. 6B). No signal for endogenous PAI-2 mRNA was detected in the 10 wild-type papillomas analyzed (data not shown). Similar results were obtained in hyperproliferative lesions after TPA termination (Fig. 6, C and D). Thus the effect of PAI-2 on transgenic papilloma formation and skin hyper-
proliferation appears to be independent of uPA expression. Interestingly, when lesions progressed to an invasive carcinoma stage, they were characterized by switching on expression of uPA (Fig. 6D) and switching off expression of the K5-PAI-2 transgene (Fig. 6C).

DISCUSSION

PAI-2 is produced by a relatively limited number of cell types, including differentiating keratinocytes. In these and other cells, the...
role of this serpin class antiprotease remains enigmatic, particularly because it has a bipotential distribution with most of the protein that accumulates in the cytosol, depending on the cell type. On the basis of in vitro experiments, it has been proposed that PAI-2 may participate in the regulation of cell death (8–11). To investigate aspects of PAI-2 function in vivo, we generated transgenic mice overexpressing PAI-2 in basal cells of the epidermis and hair follicles: this allowed us to analyze the consequences of such a dysregulation of PAI-2 expression on both skin physiology and, using a well-established protocol of chemically induced papilloma formation, skin carcinogenesis.

We have observed that K5-PAI-2 transgenic mice are highly susceptible to skin carcinogenesis elicited by an initiation-promotion protocol: in comparison with nontransgenic mice, papillomas arose earlier and developed on essentially all treated mice; most strikingly, they did not regress when promotion was discontinued. Importantly, expression of the transgene did not overcome the requirement for either initiation or promotion, suggesting that it did not mimic one or the other of these steps and thus acted in a manner different from H-ras mutations, which can replace the initiation step (24). We therefore favor the hypothesis that K5-PAI-2 transgene expression facilitated another set of events relevant to carcinogenesis. In this context, our finding that papilloma regression did not occur in transgenic mice, taken together with the observation that this is accompanied in control mice by extensive apoptosis, suggests that PAI-2 may have accelerated papilloma formation by influencing the balance between cell proliferation and cell death. In accord with in vitro results that have shown effects of PAI-2 on cell death (8–11), our observations provide the first in vivo evidence that this serpin may control apoptosis and could thereby play an important role in certain hyperproliferative disorders, either nonmalignant or malignant. In this context, it is noteworthy that overexpression of PAI-2 has been reported in human hyperproliferative skin diseases such as lupus erythematosus (26) and in several human cancers (27) including squamous cell carcinoma (28, 29). It has recently been suggested that a possible role of PAI-2 on tumor development may depend on its cellular origin because in esophageal squamous cell carcinoma, fibroblastic PAI-2 expression was correlated with a good prognosis, whereas expression in cancer cells was associated with poor prognosis (29). Our study extends these descriptive reports in that it suggests that PAI-2, when expressed in epithelial tumor cells, may promote cancer development and progression via an antiapoptotic effect.

Driven by the K5 promoter, PAI-2 was constitutively expressed in basal epidermis and hair follicle cells, as demonstrated by in situ hybridization. Why then did PAI-2 overexpression not influence the cellularity and differentiation of the epidermis and its appendages in the absence of exposure to the carcinogenic protocol? Both in vivo and in vitro studies have shown that endogenous PAI-2 is preferentially expressed in differentiating keratinocytes (15, 19); if its role, as has been proposed, is to prevent cells from premature terminal differentiation, the pathway involved may be active only in differentiating cells, and transgene-encoded PAI-2, which is expressed in the proliferating cell population, would therefore not affect the life cycle of the intact skin. During carcinogenesis, however, rapid cell proliferation may be accompanied by rapid cell death, and the proposed antiapoptotic function of PAI-2 would become manifest. A similar phenomenon has been reported in transgenic mice overexpressing Bcl-xL in the same cell population as that which expresses the K5-PAI-2 transgene. Bcl-xL is a "classical" antiapoptotic molecule, and its expression in proliferating keratinocytes does not influence the differentiation of the skin or its appendages, but it does render the mice highly susceptible to carcinogenesis (30, 31). Interestingly, rapid cell proliferation accompanied by rapid cell death does occur in normal skin in vivo: it provides a means to "sculpt" the epidermis during development (32).

Although PAI-2 can exist in two topographically different compartments (the cytosolic and extracellular compartments), PAI-2 remains mostly intracellular in keratinocytes and does not affect extracellular proteolysis. As an intracellular molecule, how can PAI-2 regulate cell death? At least two hypotheses can be envisioned. First, PAI-2 could inhibit an as yet unidentified intracellular protease because intracellular proteolysis is involved in apoptosis (33). Other serpins, such as CrmA, SPI-1, and PI-9 have been implicated in the regulation of cell death (34). CrmA, for instance, prevents cytokine processing by inhibiting caspase-1 and protects cells against Fas-1-, tumor necrosis factor-, and tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis. A putative intracellular PAI-2 target could perhaps be identified by incubating radiolabeled PAI-2 with extracts of cells induced to commit apoptosis and searching for enzyme-inhibitor complexes. Because a putative protease may be complexed mostly with endogenous PAI-2, the recent availability of PAI-2 knockout mice (35) should be helpful in this respect. Second, given that PAI-2 is a good substrate for transglutaminase (36), and because this enzyme is involved in the terminal differentiation of the epidermis (37), the high levels of PAI-2 achieved in the keratinocytes overexpressing and accumulating intracellular PAI-2 may compete with the "natural" transglutaminase substrates, thereby interfering with terminal differentiation. A similar hypothesis has been proposed by O’Brien et al. (38), who demonstrated that transgenic mice producing high levels of polyamine, an excellent substrate for transglutaminase, are highly susceptible to carcinogenesis.

Whatever the precise mechanism may be, the present study provides in vivo evidence for a role of PAI-2 in inhibiting apoptosis and for a cocarcinogenic effect of its dysregulated expression, at least in chemically induced skin carcinogenesis. Because PAI-2 is normally expressed in a limited number of cell types (39), it is unlikely to be involved in general cell apoptosis during development and cell differentiation. This may explain why PAI-2 deficiency does not alter overall development, fertility, or survival of mice (35). However, it will be of interest to determine whether overexpression or aberrant expression of PAI-2 accompanies the development of other cancerous lesions in the skin or other tissues.

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