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Extracellular proteolysis alters tooth development in transgenic mice expressing urokinase-type plasminogen activator in the enamel organ

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SUMMARY

By catalyzing plasmin formation, the urokinase-type plasminogen activator (uPA) can generate widespread extracellular proteolysis and thereby play an important role in physiological and pathological processes. Dysregulated expression of uPA during organogenesis may be a cause of developmental defects. Targeted epithelial expression of a uPA-encoding transgene under the control of the keratin type-5 promoter resulted in enzyme production by the enamel epithelium, which does not normally express uPA, and altered tooth development. The incisors of transgenic mice were fragile, chalky-white and, by scanning electron microscopy, their labial surface appeared granular. This phenotype was attributed to a defect in enamel formation during incisor development, resulting from structural and functional alterations of the ameloblasts that differentiate from the labial enamel epithelium. Immunofluorescence revealed that disorganization of the ameloblast layer was associated with a loss of laminin-5, an extracellular matrix molecule mediating epithelial anchorage. Amelogenin, a key protein in enamel formation, was markedly decreased at the enamel-dentin junction in transgenics, presumably because of an apparent alteration in the polarity of its secretion. In addition, increased levels of active transforming growth factor-β could be demonstrated in mandibles of transgenic mice. Since the alterations detected could be attributed to uPA catalytic activity, this model provides evidence as to how dysregulated proteolysis, involving uPA or other extracellular proteases, may have developmental consequences such as those leading to enamel defects.

Key words: Urokinase, Extracellular proteolysis, Tooth development, Enamel, Mouse

INTRODUCTION

Cell-cell and cell-extracellular matrix (ECM) interactions provide cells with information essential for controlling morphogenesis, cell fate, tissue-specific functions, cell migration, tissue repair and cell death. Proteolytic degradation or activation of cell surface and ECM proteins can mediate rapid and irreversible responses to changes in the cellular microenvironment (Werb, 1997) and thus play decisive roles in physiological and pathological processes, through regulation of ECM assembly, removal of excess ECM components, remodeling of ECM structure and release of bioactive fragments and growth factors. The major enzymes that cleave ECM and cell surface proteins are matrix metalloproteases (MMP) and serine proteases. As a serine protease, uPA plays an important role in extracellular proteolysis by activating plasminogen to plasmin, which in turn initiates the degradation of ECM molecules either directly or indirectly by converting pro-MMPs to MMPs (Vassalli et al., 1991). In addition, plasmin can activate or release transforming growth factor-β (TGF-β) (Taipale et al., 1992), basic fibroblast growth factor (Flaumenhaft and Rifkin, 1990) or insulin-like growth factors (Campbell et al., 1992). Besides plasminogen, hepatocyte growth factor (HGF) (Naldini et al., 1992), fibronectin (Quigley et al., 1987) and 72 kDa collagenase type IV (Keski-Oja et al., 1992) can also be direct uPA substrates. Thus, uPA-mediated proteolysis can affect ECM structure and/or modulate local availability or activity of growth factors. These events, alone or in combination, can influence the architecture and function of cells in tissues.

In view of the multiple effects that extracellular proteases in general, and uPA in particular, can have on cells and tissues, dysregulated proteolysis, through inappropriate expression of enzymes or inhibitors, for instance, could be a triggering event in a variety of pathologies. This may be particularly relevant to development and cell differentiation, since growth factors as well as interactions between cells and with the ECM are critical to organogenesis. One approach to investigate possible consequences of dysregulated proteolysis is by generating transgenic mice in which perturbations of the protease-antiprotease balance have been imposed in specific tissues. We have thus prepared transgenic mice that overexpress uPA in the epidermis, its appendages and derivatives, and have studied the resulting phenotype.

Basement membranes (BM) are highly organized ECMs providing physical support for the overlying cells, the functional activity of which often depends on BM integrity. In particular, BMs play a prominent role in the establishment and maintenance of epithelial cell polarity. Amongst epithelia, the labial enamel epithelium is unique in that, when they differentiate into secretory ameloblasts, the epithelial cells reverse their polarity: the pole of the cells that was originally basal, i.e. oriented towards the BM, becomes functionally apical and is a site of secretory processes involved in enamel deposition. Concomitant with or preceding this reorganization are the degradation of the original BM and the appearance of a BM-like ECM structure at the opposite side of the cells, which becomes functionally basal. This newly formed ECM may be required for sustaining reversed polarity of the epithelial cells. Later, during enamel maturation, ameloblasts reform a BM on the enamel surface, and the secretory polarity of the cells once again reverses.

Here we report that mice in which a uPA cDNA is controlled by the bovine keratin type-5 (K5) promoter (Ramirez et al., 1994) express uPA not only in basal epidermal keratinocytes, as expected, but also in the enamel epithelium, which elaborates enamel on its labial side. The activity of the K5 promoter in enamel epithelium suggests that keratin type-5 is produced in this tissue; this would explain why enamel hypoplasia is common in patients with epidermolysis bullosa simplex (Wright et al., 1993), a skin disease caused by mutations in either keratin type-14 or type-5 genes (Fuchs et al., 1994). K5-uPA transgenic mice have profound defects in enamel formation, presumably related to a failure to maintain or establish the appropriate BM-type structures required for correct organogenesis; by contrast, their epidermis appeared unaffected. These results demonstrate that expression of uPA in the enamel organ leads to marked alterations in the formation and structure of teeth, and hence that dysregulated proteolysis can be a cause of developmental defects.

MATERIALS AND METHODS

Generation of transgenic mice

A keratin 5 promoter-driven mouse uPA-encoding transgene was prepared as follows: An *Xba*I-*Bgl*II fragment (1670 bp) encoding mouse uPA (Belin et al., 1985) was subcloned in the *Xba*I-*Bam*HI sites of the Bluescript KS-II vector (Stratagene) to produce pBS-uPA. The *Xba*I-*Cla*I fragment from pBS-uPA was cloned into the *Eco*RI (position 1540 of rabbit β-globin genomic sequence, modified to *Xba*I-*Cla*I sites) site of pBS-β-globin plasmid, to generate β-globin uPA. This construct thus contained a β-globin intron (900-1540), the coding sequence for uPA and the polyadenylation signal (1540-2086) of the β-globin genomic sequence. The bovine keratin 5 promoter (K5) was added, by inserting a 5200 bp *Kpn*I blunted-*Not*I fragment (Ramirez et al., 1994), in the *Sac*II blunted-*Not*I site of β-globin uPA, to generate the K5-uPA transgene. A *Kpn*I-*Sal*I (8240 bp) insert was purified from this construct to produce transgenic mice by pronuclear injection of fertilized CBAJ/Bl6 F1 zygotes.

Identification of transgenic mice

This was performed by amplifying 1 μg of tail genomic DNA by 35 cycles (94°C, 55°C, 72°C, 1 minute each) of polymerase chain reaction (PCR) with the following primers: globin (GLO) 5′-GCATATAAATTCTGGCTGGCGTGG-3′ and uPA 5′-GGCCTTT-CCTCGGTAAGAGTCACC-3′. An aliquot (10 μl) of each reaction was resolved in a 1% agarose gel and amplified fragments visualised by ethidium bromide staining. To confirm the nature of the amplified fragment, part of the reaction (40 μl) was *Apa*I-digested and visualised as above. Positions of the primers and of the *Apa*I site are illustrated in Fig. 1A.

Teeth dissection and examination

Adult mice were killed by decapitation. The lower incisors and molars were gently extracted from mandibles incubated with protease K (100 μg/ml, 37°C, 3 hours), washed and processed for light or scanning electron microscopy. The labial side of incisors was examined at different magnifications.

Preparation of tissue sections

Neonatal mice (day-1 or day-3) were killed as above. Heads were fixed in 4% paraformaldehyde for routine paraffin sections or snap-frozen in liquid nitrogen for cryosections. They were sectioned sagitally until incisors were exposed. Paraffin sections (5 μm) were processed for hematoxylin-eosin staining and cryosections (10 μm) were mounted on polylysine-coated slides for in situ hybridization and immunostaining.

In situ hybridization

Constructs SP64-muk containing antisense *Pst*I-*Hin*dIII fragments of mouse uPA DNA (provided by D. Belin, Department of Pathology, University of Geneva) was linearized and transcribed to generate digoxigenin-labeled RNA probes, using a digoxigenin labeling kit (Boehringer, Germany). The probes were fragmented by alkali hydrolysis and diluted in hybridization buffer to a concentration of 100 ng/ml. Processing of cryosections, hybridization and detection was performed as previously reported (Komminoth, 1996).

Zymography

Adult transgenic mice and their wild-type littermates were anesthetized with ethrane and a piece of back skin removed using an 8 mm biopsy punch; fetal (17-18 days post coitum, p.c.) mandibles were collected from decapitated fetuses and cut through the plane of the eyes. Samples were processed for SDS-PAGE and zymography, or cryosectioned for in situ zymography. For protein extraction, the samples were homogenized in 1 (skin) or 3 (mandibles) ml 100 mM Tris, pH 8.0 / 100 mM NaCl, centrifuged (15000 revs/minute, 4°C, 15 minutes) and the supernants collected. uPA activity was detected by SDS-PAGE and zymography of electrophoretic gels and proteolysis was recorded using dark-ground illumination (Vassalli et al., 1977). In situ zymography was performed by coating a layer of indicator gel (Vassalli et al., 1977) over cryosections (10 μm); the sections were incubated in a humid box at 37°C and proteolysis visualized as above. Detection of MMP activity was performed by gelatinolytic zymography (Oliver et al., 1997).

RT-PCR

cDNA was reverse-transcribed from 2 μg total RNA from neonatal mandibles using random primers (p(dN)6, 2 mM; Pharmacia). An aliquot (1/20) of the reaction was subjected to PCR (40 cycles). The primers chosen for amelogenin are nucleotides 60-82 (sense) and 623-644 (antisense) of mouse neonatal mandible amelogenin cDNA (accession no. D31768). The primers for tuftelin are nucleotides 72-91 (sense) and 891-910 (antisense) of mouse tuftelin cDNA (accession no. G2183284). The primers for β-actin are H3 and H4 (Minty et al., 1982); they amplify a 231 bp fragment. Each reaction cycle was as follows: 50 seconds at 95°C, 50 seconds at 68°C, and 60 seconds at 72°C for amelogenin; 50 seconds at 95°C, 50 seconds at 62°C, and 30 seconds at 72°C for tuftelin; 40 seconds at 95°C, 40 seconds at 55°C and 40 seconds at 72°C for β-actin. Unless indicated, reagents were from Boehringer Mannheim.

Immunohistology

The cryosections were fixed 4 minutes in 4% paraformaldehyde, washed in PBS and blocked 30 minutes in 8% milk. Rabbit antisera against amelogenin (a gift from Dr Magarita Zeichner-David, Center for Craniofacial Molecular Biology, School of Dentistry, University of Southern California) or laminin-5 (a gift from Dr Robert E Burgeson, Cutaneous Biology Center, Massachusetts General Hospital, Harvard Medical School) were 1 to 400 diluted in 2% milk and applied to the sections for 2 hours at room temperature. After washing, sections were incubated with FITC- (for antilaminin-5) or peroxidase- (for amelogenin) labeled anti-rabbit immunoglobulin antibodies (Sigma). The peroxidase activity was revealed using 0.03% H_2O_2 and 0.05% diaminobenzidine.

Detection of active TGF-β

The MLEC cell line (Abe et al., 1994) was provided by Dr Daniel B. Rifkin, New York University Medical Center. The cells were cultured as reported (Abe et al., 1994). After having grown to 60% confluence in 6-well tissue culture plates, the cells were shifted to DMEM without FCS and incubated in triplicate in presence of mandible extracts. Extracts were prepared as follows: fetal mandibles cut through the plane of the eyes were immediately homogenized in 3 ml ice-cold PBS containing 1 mM PMSF and 76 mU/ml aprotinin (Sigma). After centrifugation (15000 revs/minute, 15 minutes at 4°C), supernatants were collected and 100 µl were added to MLEC cultures (1 ml); as a control, 100 µl of the homogenization buffer were added to parallel cultures. After 24 hours of culture, the cells were washed twice in ice-cold PBS and lysed on ice in 200 µl of 1x lysis buffer for luciferase assay (Promega). The lysates were centrifuged (15000 revs/minute 15 minute at 4°C), and 30 µl of supernatant were mixed with an equal volume of luciferase substrate (Promega). Luciferase activity was immediately measured using a luminometer.

RESULTS

Characterization of transgenic mice

Three individuals (no. 119, 126 and 130) bearing the K5-uPA transgene (Fig. 1A) were identified amongst 150 candidate pups, by dot-blot hybridization using rabbit β-globin intron cDNA as probe (not shown). Presence of the transgene was confirmed by PCR amplification of a fragment of the anticipated 508 bp size; digestion of this fragment at a unique *Apa*I site in the β-globin intron yielded the expected bands of 366 and 142 (Fig. 1A,B). These three individuals transmitted the transgene to their offsprings; they were used as founders to establish independent transgenic lines by back-crossing with CBAJ mice. To analyze the specificity of transgene expression, in situ hybridization was performed on cryosections of back skin, using a digoxigenin-labeled mouse uPA cRNA probe. uPA mRNA was detected in the basal layer of epidermis and in the outer root sheath of hair follicles in transgenic mice but not in their wild-type littermates (Fig. 1C), indicating that, as expected, transgene expression was targeted to these sites by the promoter used and that there is no expression of endogenous uPA in epidermis under

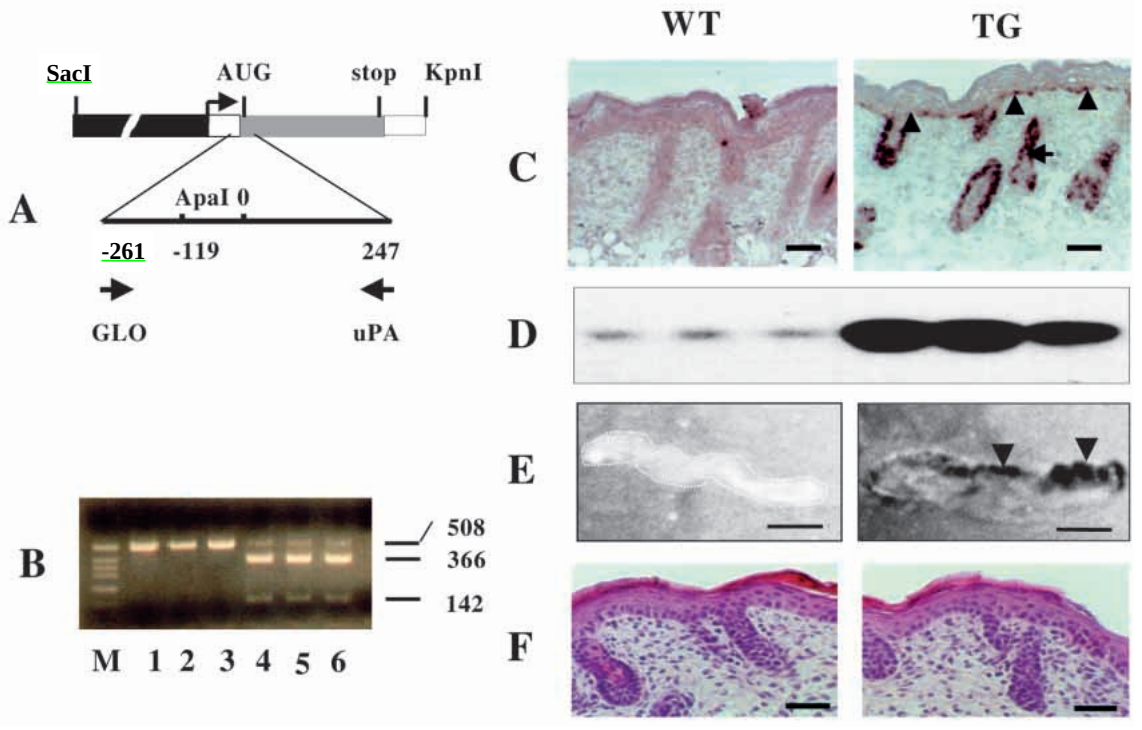

Fig. 1. Structure and expression of the K5-uPA transgene. (A) From left to right, the components of the transgene are: bovine keratin type-5 promoter (black), rabbit β globin intron (white), murine uPA cDNA (grey) and rabbit (globin poly(A) addition signal (white). PCR primers used to analyze transgene integration are indicated (GLO and uPA); they amplify a 508 bp fragment. An *Apa*I site located in the rabbit (globin intron (–119) is indicated. (B) PCR-amplified fragments from DNA prepared from the three transgenic lines were resolved in a 1% agarose gel either directly (lanes 1-3, 508 bp fragment) or after *Apa*I digestion (lanes 4-6, 366 and 142 bp fragments) and visualized by ethidium bromide staining. As a marker (M), the *Hin*fI-digested fraction of a 1 kb ladder is shown. (C) In situ hybridization shows the presence (brown staining) of uPA mRNA in the basal epidermal layer (arrowhead) and the outer root sheath of hair follicles (arrow) in transgenic (TG) but not in wild-type (WT) mice. Bar: 100 µm. (D) SDS-PAGE zymography reveals high levels of uPA activity in skin extracts from the three transgenic lines, but not in samples from their wild-type littermates. No lysis was observed when the zymography was performed in the absence of plasminogen (not shown). (E) In situ zymography reveals uPA activity (arrows) along the epidermis of transgenic but not of wild-type mice. The wild-type skin specimen is outlined by the dotted line, with epidermis located at the upper surface. No lysis was observed when the zymography was performed in the absence of plasminogen (not shown). Bar: 50 µm. (F) Hematoxylin-eosin staining of paraffin sections from neonatal back skin. The epidermis and its appendages appear similar in both specimens.

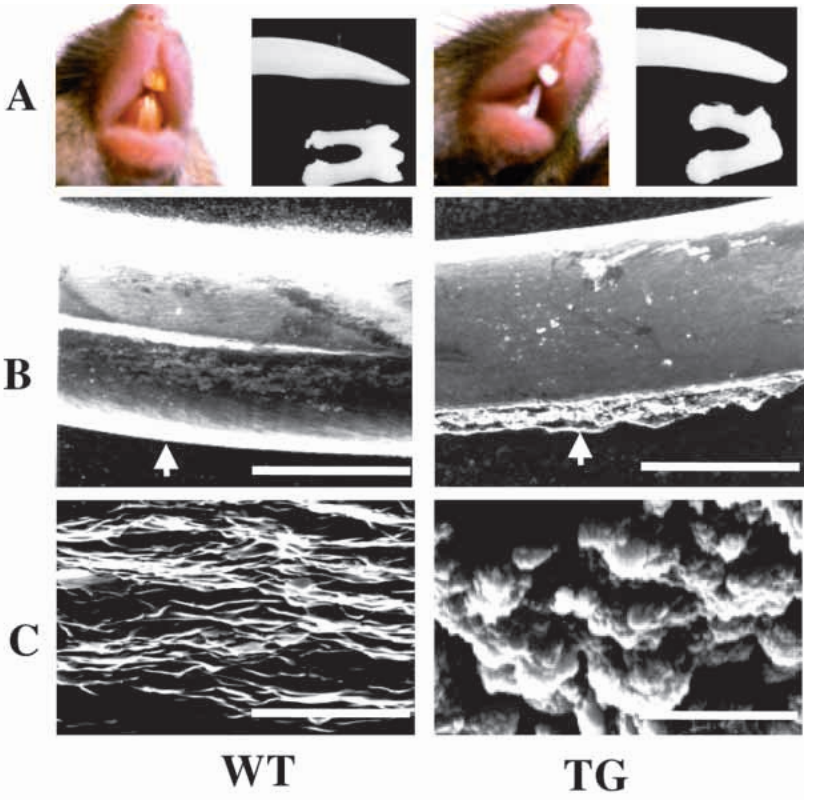

Fig. 2. Incisors and molars in wild-type (WT) and transgenic (TG) mice. (**A)** In TG, incisors are always chalky-white and fragile; in the present individual, one incisor is broken.; both incisors and molars have blunted tips. WT incisors are yellow-brown and the tips of both incisors and molars are sharp. (B,C) Scanning electron micrographs of the labial surface (arrow) of incisors showing a smooth surface in WT and a granular surface in TG at low (B) or high (C) magnification. In contrast, the lingual (upper) surface appears similar in both specimens (B). Bar, 500 μm in B and 20 μm in C.

physiological circumstances. A corresponding sense probe did not yield a signal in transgenic or wild-type mice (not shown), confirming the specificity of the signal detected in transgenics with the antisense probe. To determine if the transgene-encoded protein was functional, skin extracts were analyzed by SDS-PAGE and zymography (Fig. 1D). Comparable high levels of uPA activity were detected in samples from the three transgenic lines, while samples from wild-type mice contained merely detectable uPA, which might have derived from blood. In situ zymography (Fig. 1E) confirmed the presence of net uPA activity in transgenic, and not in wild-type, epidermis. Histological analysis of skin did not reveal detectable differences in epidermal structure or thickness, or in the structure or density of hair follicles, between wild-type and

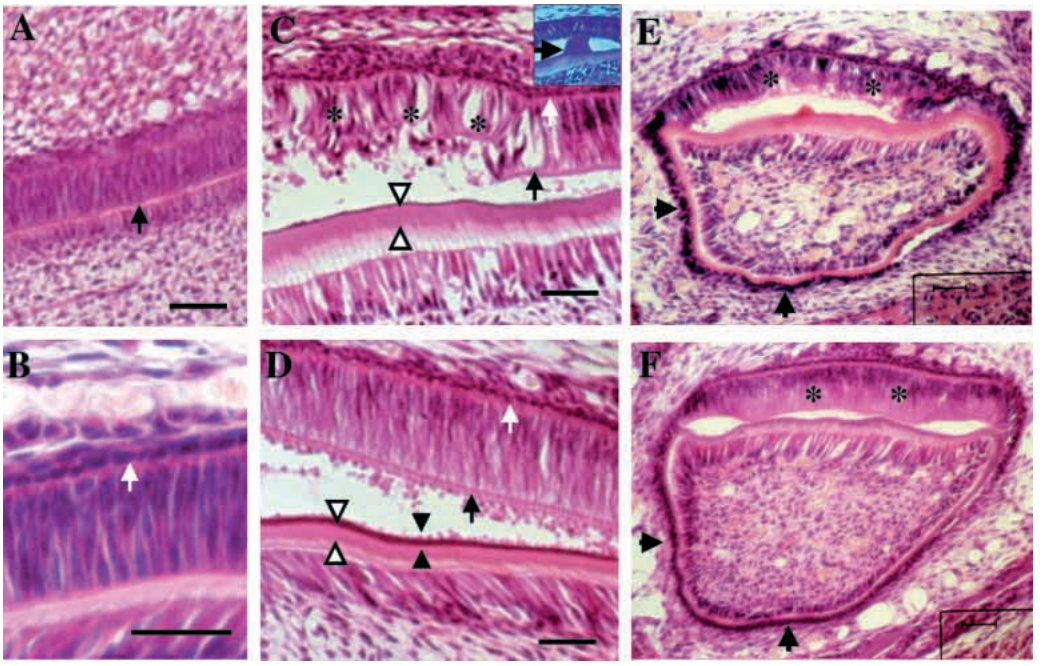

Fig. 3. Hematoxylin-eosin staining of paraffin sections from transgenic (A,B,C,E) and wild-type (D,F) enamel organs. (A) A typical basal BM (arrow) is evident at the preameloblast stage. (B) A BM-like structure (arrow) forms along the apical pole of preameloblasts when they start to differentiate into ameloblasts. (C) Disintegration of the basal BM (black arrow), disruption of ameloblasts (asterisks), marked reduction or absence of regular enamel deposited on dentin surface, frequent occurrence of large crystals (insert), but apparently normal dentin (pink layer between two white arrowheads) are typical of the transgenic mice. (D) Normal ameloblasts, dentin (white arrowheads), enamel (purple layer between two black arrowheads and apical (white arrow) BMs in a wild-type mouse. (E,F) Upper incisors seen at low magnification. Disorganization of enamel epithelium on the labial side (asterisks) is evident in transgenic (E) as compared to wild type (F), in contrast, the lingual side epithelium seems normal (arrows). Bars: 100 μm (A,C-F); 60 μm (B).

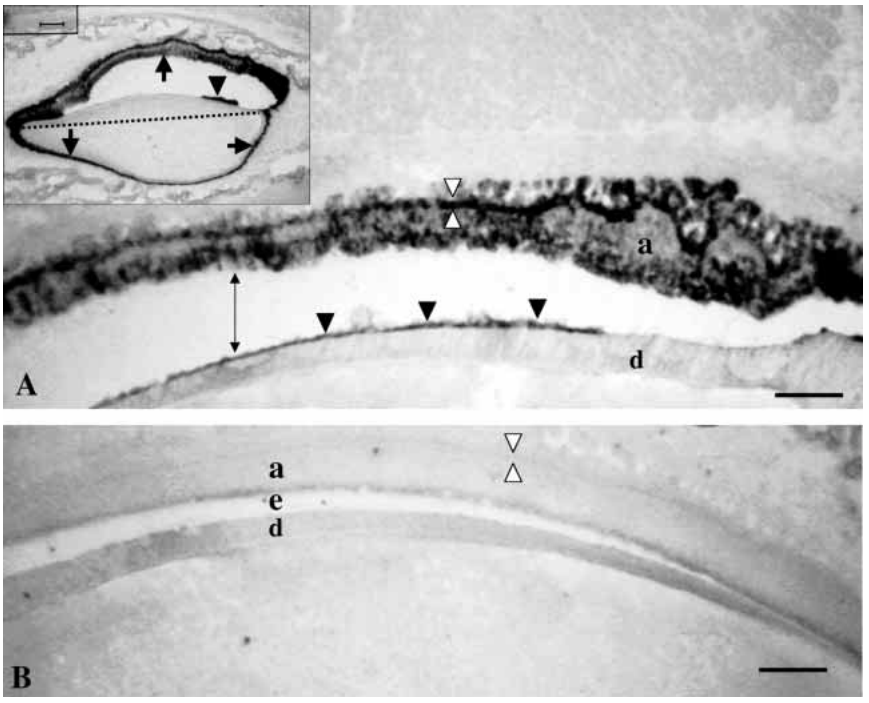

Fig. 4. In situ hybridization of uPA mRNA in 3-day-old enamel organs. Using a digoxigenin-labelled antisense uPA cRNA probe, uPA mRNA is revealed by the alkaline phosphatase reaction product (black). (A) In transgenic mice, ameloblasts (a) and stratum intermedium (between white arrowheads) are positive; positive staining (black arrowheads) overlying the dentin (d) is due to the presence of fragments from broken ameloblasts. The insert is a low magnification of a transgenic enamel organ showing the presence of uPA mRNA (arrows) in enamel epithelium on both the labial (above the dashed line) and lingual (below the dashed line) sides; the black arrowhead indicates positive staining in dentin-adherent ameloblast fragments. Notice the wide space (↔) between ameloblasts and dentin and the apparent absence of enamel. (B) In wild-type mice, no uPA mRNA is detected in the ameloblasts (a). Enamel (e) and dentin (d) are indicated. Bar: 100 μm.

transgenic mice (Fig. 1F). Transgenic mice were indistinguishable from their littermates in terms of body size or behaviour before weaning. After weaning, transgenics were occasionally found wasting, presumably owing to their fragile incisors (Fig. 2A); this status was improved by feeding with soft food. No differences in life span or fertility were observed between wild-type and transgenics over a two-year period of observation. Since all transgenic individuals from the three lines had the phenotype reported below, the results obtained with line #130 were selected for a detailed description.

Abnormal incisors in transgenic mice

The gross appearance of incisors in adult wild-type and K5-uPA transgenic mice is illustrated in Fig. 2A. Strikingly, while the incisors of wild-type mice were always yellow, the incisors of transgenics were consistently chalky-white and frequently broken. This colour difference became increasingly remarkable with age; it could be used to predict the genotype of littermates aged 3 weeks and above with 100% accuracy. The yellow colour of rodent incisors derives from enamel pigmentation before eruption (Selvig and Halse, 1975; McKee et al., 1998). Therefore, white incisors in transgenics indicate a failure of this pigmentation and thus suggest a developmental defect in enamel formation. The frequent occurrence of broken incisors is in accord with this hypothesis. By scanning electron microscopy, the labial surface of wild-type incisors appeared smooth, while that of transgenic incisors was granular, consisting of irregularly sized crystals (Fig. 2B,C); by contrast, no obvious difference was observed in the appearance of the lingual surfaces (Fig. 2B). In rodent incisors, only the labial side is covered by enamel; the selective alteration of this region in transgenics thus reinforces the notion of a defect in enamel formation. This defect renders transgenic teeth particularly susceptible to erosion, resulting in blunt-tipped incisors (Fig. 2A). Although molar enamel formation was not investigated in detail, the blunted crown of molars (Fig. 2A) suggests that it is also affected in transgenic mice.

Histological alteration of ameloblasts in transgenic mice

Ameloblasts, the cells directly responsible for enamel formation, differentiate from the labial enamel epithelium. Defects in enamel could thus result from a perturbation of this differentiation process. The morphological differentiation of the epithelium in transgenic mice appeared normal up to the ameloblast stage: prior to becoming preameloblasts, the cells had a typical BM along their structural basal pole (BBM) (Fig. 3A); the preameloblasts then appeared to establish a BM-like structure at their structural apical pole (ABM) (Fig. 3B) and further differentiated into ameloblasts, which could be

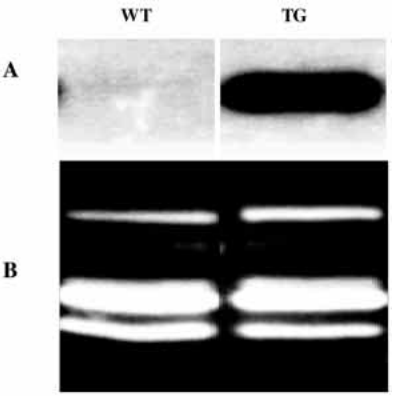

Fig. 5. Zymographic analysis of uPA (A) and metalloproteases (B) in mandible extracts of fetal (~17 days p.c.) wild-type (WT) and transgenic (TG) mice. uPA is detected in transgenics only. Three species of gelatin-degrading activities are present in both transgenic and wild-type mice, with no differences in either the type or the amount of enzymes; the two major bands have an apparent M_r of 94 and 72 kDa.

recognized by their elongated cell height and apical nuclear polarization. Striking differences were observed when comparing transgenic (Fig. 3C,E) and wild-type (Fig. 3D,F) labial epithelium ameloblasts, however. In particular, disruptions of the ameloblast layer were conspicuous in transgenics; these alterations were associated with, and may have been secondary to, focal disintegrations of the ABM and BBM (Fig. 3E). In wild-type mice, by contrast, the ameloblasts were aligned between two continuous fibrous structures (BMs) lining their basal and apical poles, their nucleus was apically polarized, and a regular layer of forming or completed enamel could be seen overlying the dentin (Fig. 3F). At the same stage in transgenics (Fig. 3E), the enamel layer was absent or merely visible, but large crystals were frequently observed (Fig. 3C,E insert). The stratum intermedium of transgenic incisors appeared disorganized in places (Fig. 3E), suggesting that the ABM may also be required for maintenance of the architecture of this tissue. In contrast to the labial epithelium, the lingual epithelium, which does not differentiate into ameloblasts and does not form enamel, was morphologically normal in transgenics (Fig. 3E). Despite these alterations in ameloblast structure and enamel deposition, dentin formation appeared unaffected in transgenic mice (Fig. 3C,E).

The K5-uPA transgene is expressed in the enamel organ

The distinctive features of teeth described above were observed in the three independent transgenic lines, indicating that the phenotype is not due to transgene integration-induced mutations or to genetic differences other than transgene expression. They suggest that the transgene may be active in the enamel organ during incisor formation. This was verified by in situ hybridization: uPA mRNA was detected in internal (labial and lingual, Fig. 4A) and external (not shown) enamel epithelia as well as in the stratum intermedium (Fig. 4A) of neonatal transgenic mice (Fig. 4A), but not in their wild-type littermates (Fig. 4B). By places, the dentin in transgenic incisors was covered by material positive for uPA mRNA; this appeared to represent ameloblast fragments, reinforcing the notion that the ameloblast layer is disrupted in transgenics. Zymographic analysis of mandible extracts confirmed the presence of uPA in transgenic but not in wild-type mice (Fig. 5A). Transgene-encoded uPA mRNA could be detected in specimens from younger animals, before morphological alterations became conspicuous (not shown), suggesting that uPA expression could be the cause, rather than the consequence, of the changes in enamel organ structure.

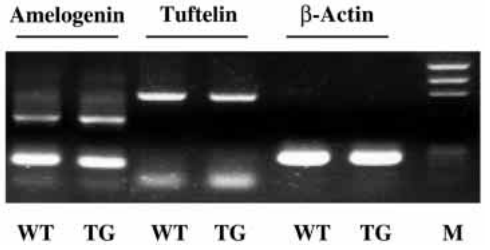

Fig. 6. RT-PCR analysis of amelogenin and tuftelin gene expression in mandibles of day-1 wild-type (WT) and transgenic (TG) mice. Shown are amplified fragments resolved in a 1% agarose gel and revealed by ethidium bromide staining. With the primers used, a 584 bp amelogenin fragment (upper) is amplified; the smaller ~200 bp fragment presumably derives from a different splicing form of the mRNA. For tuftelin mRNA, the expected 838 bp fragment is amplified. As an internal control, a 231 bp β-actin fragment is amplified. The amplification profiles are the same for both WT and TG. ΦX 174 DNA/*Hae*III was loaded as a marker (M).

Expression of amelogenin and tuftelin

Amelogenin and tuftelin are two ameloblast-secreted proteins involved in enamel formation. The enamel defect could be due to decreased expression of either of these two genes as a consequence of ameloblast dysfunction. This possibility was explored by RT-PCR on total RNA from neonatal wild-type and transgenic mice. For both amelogenin and tuftelin mRNAs, a similar pattern of amplification was obtained in the two samples (Fig. 6), suggesting that transcription of these genes was unaffected. For amelogenin, in addition to the expected fragment, a smaller fragment was also amplified, which had not been anticipated according to the reported sequence (accession D31768); this amplification appeared specific, since this fragment was not obtained using liver or testis cDNAs, and it may be due to a different splicing form of amelogenin mRNA. We next examined the presence of amelogenin by immunostaining of tissue sections. In wild-type mice, amelogenin was revealed as a regular layer along the enamel-dentin junction (EDJ), and as discrete intracellular spots (Fig. 7A). In transgenics, the amelogenin layer at the EDJ was fragmented or absent (Fig. 7C), and discrete amelogenin spots were seen extracellularly in the apical area of ameloblasts and the stratum intermedium (Fig. 7D), indicating that polarity of amelogenin transport and/or secretion may be altered; the protein was also detected in the basal region of ameloblasts, suggesting reabsorption or altered secretion. Taken together, these observations indicate that accumulation of amelogenin at the EDJ was markedly decreased in K5-uPA transgenics, through a process that did not involve repression of transcription of the amelogenin gene. Amelogenin is a regulator of hydroxyapatite crystal growth, size and orientation (Zeichner-David et al., 1995). The large crystals at the EDJ of transgenic mice (Fig. 3C insert, E) are thus compatible with a defect in amelogenin accumulation.

Transgene expression perturbs basement membrane integrity

The integrity of the BM is important for the structure and function of epithelia. Considering that BM components can be cleaved or degraded by uPA-catalyzed proteolysis, the modified phenotype of ameloblasts suggests that the enamel organ BMs may have been altered in transgenic mice. To test this hypothesis, we determined the distribution of laminin-5 (LN-5), a BM component that mediates attachment of epithelial cells, in the enamel organ (Yoshiba K., et al., 1998; Yoshiba N., et al., 1998), as well as in the skin, which is not affected by transgene expression and thus serves as a control. In epidermis, LN-5 was conspicuous in the BM and the staining pattern was indistinguishable between wild type (Fig. 8A) and transgenics (Fig. 8B). By contrast, in the enamel organ, there was a marked difference in LN-5 distribution depending on the genotype. In wild-type mice, LN-5 was distributed throughout the ameloblast layer and along the plane of basal and apical BMs (Fig. 8C). In transgenics, LN-5 in the ameloblast layer appeared restricted to the basal pole; moreover, LN-5 was nearly totally missing in the region of the

apical BM and markedly reduced in the basal BM. LN-5 was also detected along the BM of the external enamel epithelium in wild-type mice (Fig. 8C); in transgenics (Fig. 8D), the labeling of this structure was clearly decreased. These observations confirm that the organization of the enamel organ BMs was disturbed in transgenic mice, presumably as a consequence of uPA-catalyzed proteolysis. They support the idea that the morphological alterations and dysfunction of transgenic ameloblasts may be secondary to a degradation of LN-5 and/or other BM constituents.

MMP activity is not upregulated in K5-uPA transgenic mice

One aspect of uPA-mediated proteolysis that may be relevant to the transgenic phenotype is the plasmin- or uPA-catalyzed activation of certain pro-MMPs to active enzymes, which in turn can cleave LN-5 and other BM proteins. To investigate whether MMPs are upregulated by uPA expression in transgenic mice, we analyzed whole mandible extracts by gelatinolytic zymography. Despite high levels of uPA activity in transgenic mandibles (Fig. 6A), no difference in either the type or the amount of detectable MMPs was observed between transgenic and wild-type samples (Fig. 6B).

Upregulation of active TGF-β in K5-uPA transgenic mice

TGF-β and its cognate receptor are expressed in the enamel organ (Lunstrum et al., 1995). Given that TGF-β is secreted in a latent form that can be activated by plasmin (Lyons et al., 1992), active TGF-β may be increased in the transgenic enamel organ. This hypothesis was verified using an MLEC cell line carrying a luciferase-encoding reporter gene placed under the control of a TGF-β responsive promoter. Luciferase activity in cells incubated for 20 hours in the presence of transgenic mandible extract was 2.3-fold that in cells incubated with extract from their wild-type littermates (Fig. 9), indicating upregulation of active TGF-β in the enamel organ of K5-uPA transgenic mice.

DISCUSSION

The present transgenic model was established to study the role of dysregulated uPA-catalyzed extracellular proteolysis in the skin and its appendages. The abnormal teeth had not been anticipated. This observation led to the finding that transgene-encoded uPA is expressed in enamel epithelium, indicating that the K5 promoter is active in this tissue. Thus, the keratin type-5 gene may also be active in enamel epithelium. Since keratins type-5 and type-14 are in general expressed concomitantly, this speculation is supported by the report that enamel epithelium expresses keratin type-14 (Tabata et al., 1996b). This would explain why enamel development is affected in patients with epidermolysis bullosa (EB) simplex (Wright et al., 1993), a cutaneous disease resulting from mutations in either type-5 or type-14 keratins (Fuchs et al., 1994). In this context, it will be interesting to examine teeth development in mice expressing mutant keratin type-14 (Lloyd et al., 1995). Other forms of EB, EB junctional and EB dystrophic, which are caused by mutations in LN-5 (Christiano et al., 1996, 1997), its receptor integrin α6β4 (Vidal et al., 1995; Pulkkinen et al., 1994) or type VII collagen (Christiano et al., 1996, 1997), also result in enamel hypoplasia. Functionally, the different molecular defects that cause the different types of EB give rise to similar consequences, i.e. a breakdown of BM-epithelium interactions. Given the similarity of these interactions in skin and enamel organ, any perturbation of such interactions that affects the skin might also cause enamel defects.

As a serine protease, uPA is a catalyst of extracellular proteolysis. By interacting with a plasma membrane protein, the uPA receptor, it also activates signal transduction pathways (Dear and Medcalf, 1998). However, this growth-factor-type activity is not involved in the phenotype of K5-uPA transgenic mice: ameloblasts do not express uPA receptor and incisors appear normal in another transgenic line (K5-ATF) that

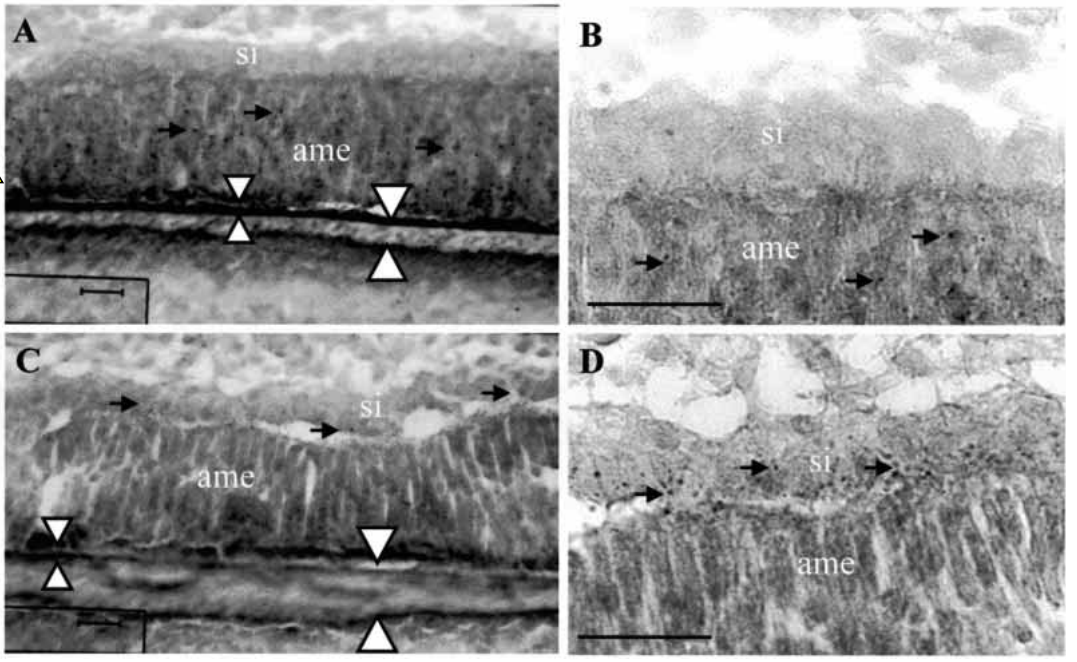

Fig. 7. Immunohistological detection of amelogenin. In wild-type mice (A), amelogenin is detected as discrete spots (arrows) within the layer of ameloblasts (ame) and as a dense layer (arrowheads) over dentin (bigger arrowheads) at the EDJ. In transgenic mice (C), discrete spots are seen in the apical extracellular space (arrows); at the EDJ, amelogenin deposition is strongly reduced and fails to form a dense, continuous layer (smaller arrowheads) over dentin (arrowheads). (B,D) Higher magnifications of A and C, showing amelogenin deposits at the interface between ameloblasts and stratum intermedium (si) in transgenic (D) but not in wild-type (B) mice. Note the presence of an extracellular space between ameloblasts and stratum intermedium in transgenic mice (D). Bar: 25 μm.

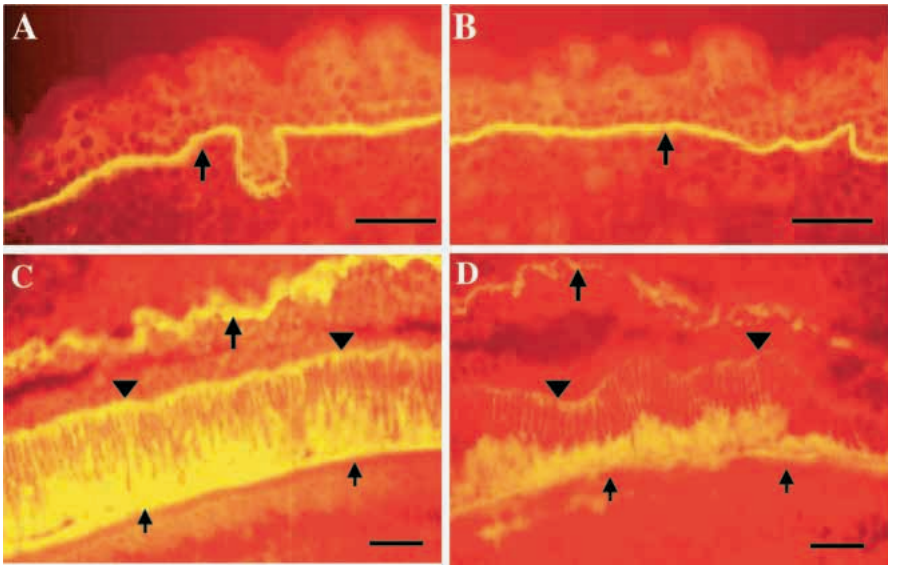

Fig. 8. Immunofluorescence detection of LN-5 in skin and enamel organ. In skin, LN-5 is present along the epidermal BM (arrow), and there is no difference between wild-type (A) and transgenic (B) mice. In the enamel organ of wild-type mice (C), LN-5 is distributed within the ameloblast layer and the basal (short arrows) and apical (arrowheads) BMs, as well as along the BM of the external enamel epithelium (long arrow). (D) In transgenic mice, LN-5 appears polarised towards the basal pole of ameloblasts and is markedly reduced in the BMs (arrows). Bar: 100 μm.

expresses a truncated form of uPA containing the receptor-binding amino-terminal fragment of the protein but lacking its catalytic domain (unpublished observations). Therefore, the altered phenotype of teeth in K5-uPA transgenic mice can be attributed to the catalytic activity of uPA. The uPA-mediated conversion of plasminogen to plasmin can result in widespread extracellular proteolysis, and thereby lead to cleavage/degradation of certain BM components, improper processing or removal of enamel proteins, or activation of certain growth factors.

In the present report, we show that LN-5, a BM component that anchors epithelial cells to BMs and is abundant in ameloblasts and in the two associated BMs of wild-type mice, is almost completely missing in the apical BM and markedly reduced in the basal BM of K5-uPA transgenic mice. Given the importance of interactions between LN-5 and its receptor (integrin α6β4) in stabilizing cell architecture (Baker et al., 1996) and in signal transduction (Giancotti, 1997), the decrease in LN-5 could have a profound impact on the structure and function of the enamel epithelium. Indeed, this decrease was associated with marked morphological and functional alterations of ameloblasts, in particular with respect to cell polarity and amelogenin transport and deposition at the EDJ. Changes in polarity of secretion due to BM modifications could provide a link between the observed changes in LN-5 and in amelogenin distribution in K5-uPA transgenics. In addition, the enamel defects associated with the default in amelogenin deposition at the EDJ of transgenic mice are similar to those resulting from mutations in the amelogenin gene (Lagerstrom et al., 1991; Lench et al., 1994); this is consistent with the key role proposed for amelogenin in enamel formation and maturation.

Enamel proteases are required to process amelogenins secreted in the ECM and later, during the maturation stages of enamel formation, to remove proteins including amelogenins from the mineralizing matrix. Different enamel proteases have been described and characterized as serine proteases (amelogenase) and metalloproteases (Zeichner-David et al., 1995). Interactions between transgene-encoded uPA and/or plasmin and enamel proteases could play a role in the observed phenotype, for instance through catalytic amplification processes. The reported stage-specific secretion of enamel proteases during enamel formation could also explain why disorganization of the enamel organ epithelial cells was not observed before the ameloblast stage. Proteolysis generated by uPA might also directly cause the premature and/or excessive degradation of enamel proteins, thus contributing to the defects in amelogenin at the EDJ and in enamel formation. Since enamel serves as functional substratum to anchor ameloblasts, failure to establish an enamel layer can in turn aggravate the disorganization and dysfunction of ameloblasts. The availability of recombinant murine amelogenin M179 (Simmer et al., 1994) should allow to explore some of these possibilities.

Cytokines of the TGF-β family can profoundly affect the function of cells in tissues, including the protease-antiprotease balance critical to morphogenetic events such as angiogenesis

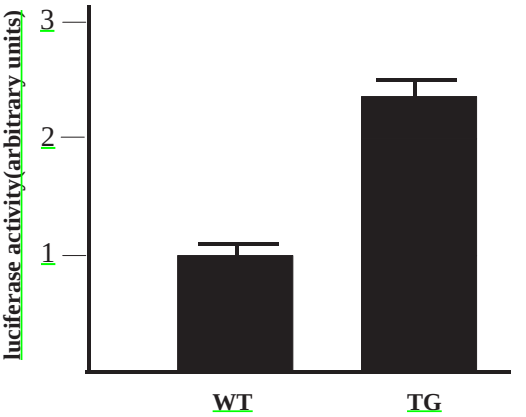

Fig. 9. Quantitation of active TGF-β. The TGF-β-responsive cell line MELC containing a luciferase-encoding reporter gene was used. The cells were incubated with fetal (~17 days p.c.) mandible extracts or extraction buffer for 20 hours and luciferase activity was measured. The basal level (extraction buffer) was subtracted; the activity in cells incubated with extract from wild-type mandibles was set as 1. The results are the average from two individuals for each genotype (wild-type, WT; transgenic, TG).

(Pepper et al., 1990). Such cytokines are produced in the enamel organ and preameloblasts express TGF-β receptors (Ruch et al., 1995). Mandibular extracts from K5-uPA transgenic mice contained elevated levels of active TGF-β, presumably through increased activation of the latent form of the cytokine by uPA-catalysed plasmin generation. Upregulation of active HGF could also be involved in the transgenic phenotype: ameloblasts express receptors for HGF (Tabata et al., 1996a) and the HGF precursor can be directly converted to the active cytokine by uPA (Naldini et al., 1992). It is thus possible that altered cytokine levels, alone or together with BM modification, play a part in the phenotype resulting from expression of the K5-uPA transgene.

The K5 promoter-driven uPA-encoding transgene was also expressed in basal keratinocytes, as expected. However, enhanced uPA expression in these cells appeared not to affect skin development. At least four specific features of the labial enamel epithelium can be considered to explain such a difference in the consequences of transgene expression. First, uPA is secreted as a zymogen that may be activated in the vicinity of ameloblasts by enzymes involved in processing or degradation of enamel proteins. Second, since ameloblasts and stratum intermedium are closely apposed cell layers that both express the transgene-encoded enzyme, the local concentration of uPA may be sufficient to directly affect BM integrity without the involvement of plasmin; relatively high levels of uPA can directly cleave fibronectin (Quigley et al., 1987), another component of BMs (Mosher et al., 1992), and activate collagenase type IV (Keski-Oja et al., 1992). Since type IV collagen is a major component of BMs, local activation of type IV collagenase will influence BM integrity. Similarly, although neither uPA nor plasmin cleave LN-5 at moderate concentrations (Giannelli et al., 1997), high local enzyme concentrations in the vicinity of BMs may be effective in its degradation. Third, LN-5 reportedly anchors ameloblasts to the enamel matrix in a lamina-free mode (Sahlberg et al., 1998). This ‘naked’ configuration, in contrast to that which prevails in typical BMs (e.g. the epidermal BM), may render the protein particularly susceptible to proteolysis. Also, LN-5 is the major and perhaps sole mediator of the association between ameloblasts and the enamel matrix (Hormia et al., 1998); degradation of LN-5 along the basal pole of the cells would thus disrupt this association, explaining why the space between ameloblasts and the EDJ is consistently wider in transgenics and why disorganization of ameloblasts is frequently most prominent in their basal region. Finally, the elongated shape of ameloblasts may render them particularly susceptible to mechanical insult; this is reminiscent of the notion that keratin mutation-induced cell breaks are only observed when cells take a columnar (in vivo) but not a flattened (in vitro) shape (Fuchs and Coulombe, 1992). These four features, alone or in combination, may explain why ameloblasts are more susceptible to expression of transgene-encoded uPA than are keratinocytes. This explanation may also apply to the lingual enamel epithelium, which has much in common with basal keratinocytes and does not appear to be affected by expression of the transgene.

An extracellular structure resembling a BM was observed between ameloblasts and stratum intermedium; although such a structure has been described before, its presence remains controversial (Pannese, 1964) and its nature remains to be documented. It was particularly evident upon anti-LN-5 staining, which revealed a continuous line along the apical pole of ameloblasts. The presence of this structure was associated with specific stages of enamel epithelium differentiation: it first appeared at the preameloblast stage, was present along secretory phase ameloblasts and disappeared when these cells entered the maturation phase. We hypothesize that this structure may be involved in the reversal of polarity of secretory ameloblasts: it could function as a BM to anchor the reversed organization of cell structure at this stage. This speculation is supported by our observation that, in K5-uPA transgenic mice, the disorganization of ameloblasts is most prominent in those regions of enamel epithelium where LN-5 deposition between ameloblasts and stratum intermedium is strongly reduced or absent.

The mechanism that causes enamel defects in K5-uPA transgenic mice may involve simultaneous or sequential combinations of the different potentially pathogenic changes summarized above. Given the broad spectrum of effects that can be ascribed to extracellular proteolysis, and in particular to the plasminogen activators/plasmin system, it may be difficult to conclusively unravel the molecular pathways involved. However, we can speculate on the developmental stage of the enamel organ at which such changes are most likely to be decisive for the observed phenotype. Before dentinogenesis, molecules (e.g. cytokines) secreted by the enamel epithelium diffuse to the underlying odontal mesenchyme and induce odontoblast differentiation and dentin formation. Later, dentin serves as a physical and chemical barrier between the enamel epithelium and the odontal mesenchyme. The integrity of dentin in our transgenic mice strongly suggests that the most relevant uPA-induced pathogenic changes occurred after and presumably independently of dentin formation. In addition, the normal differentiation of the transgenic enamel epithelium up to the ameloblast stage further supports our conclusion that it is the perturbed structure and function of the secretory stage ameloblasts that leads to the enamel defect observed in K5-uPA transgenic mice.

In conclusion, the present transgenic mouse model has demonstrated the detrimental effects that can result from alterations in the extracellular proteolytic balance during development. It has also pointed to the disruption of BMs and the ensuing morphological and functional disorganization of epithelial tissues as mediators of such developmental defects. It will be interesting to explore the possibility that spontaneous developmental defects are caused by dysregulated extracellular proteolysis, which may result from mutations in genes that encode the enzymes, their inhibitors and cofactors, or that control their expression.

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