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Lactational competence and involution of the mouse mammary gland require plasminogen

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SUMMARY

Urokinase-type plasminogen activator expression is induced in the mouse mammary gland during development and post-lactational involution. We now show that primiparous plasminogen-deficient ($Plg^{-/-}$) mice have seriously compromised mammary gland development and involution. All mammary glands were underdeveloped and one-quarter of the mice failed to lactate. Although the glands from lactating $Plg^{-/-}$ mice were initially smaller, they failed to involute after weaning, and in most cases they failed to support a second litter. Alveolar regression was markedly reduced and a fibrotic stroma accumulated in $Plg^{-/-}$ mice. Nevertheless, urokinase and matrix metalloproteinases (MMPs) were upregulated normally in involuting glands of $Plg^{-/-}$ mice, and fibrin did not accumulate in the glands. Heterozygous $Plg^{+/-}$ mice exhibited haploinsufficiency, with a definite, but less severe mammary phenotype. These data demonstrate a critical, dose-dependent requirement for Plg in lactational differentiation and mammary gland remodeling during involution.

Key words: Tissue remodeling, Plasminogen deficient mice, Mammary gland involution, Urokinase, Entactin, Metalloproteinases

INTRODUCTION

The mammary gland undergoes extensive, but finely controlled tissue remodeling throughout its growth and development. During post-pubertal maturation of the ductal tree, a variety of proteinases, growth factors and integrins are expressed in a well-regulated spatial and temporal pattern (Ossowski et al., 1979; Busso et al., 1989; Robinson et al., 1991; Coleman-Kmacik and Rosen, 1994; Witty et al., 1995; Faraldo et al., 1998; Thomasset et al., 1998). Synthesis of most proteinases ceases during late pregnancy and lactation. After weaning, the mammary gland is again remodeled in preparation for the next pregnancy through a complex and well-regulated cellular program. This process of involution involves the collapse of alveolar structures, removal of secretory epithelial cells by programmed cell death, phagocytosis by macrophages, proteolytic degradation of basement membranes, and stromal remodeling. Consequently, most of the differentiated epithelial cells disappear and an adipocyte-rich stroma, in which the resting ductal system is embedded, reappears (Lascelles and Lee, 1978; Walker et al., 1989; Strange et al., 1992; Talhouk et al., 1992; Marti et al., 1994; Lund et al., 1996). After 10-15 days, the structure of involuting glands approaches that of resting virgin glands (Lascelles and Lee, 1978).

Based on these changes, post-lactational involution can be divided into two distinct phases. The initial phase is characterized by programmed cell death of the differentiated epithelial cells and induced expression of Bax, p53 and clusterin (Lund et al., 1996; Li et al., 1997; Jerry et al., 1998). This is followed by a second phase with extensive tissue remodeling and a characteristic spatial and temporal expression pattern of a number of extracellular proteinases (Lund et al., 1996). These include the matrix metalloproteinases (MMPs) stromelysin-1 (Str1), stromelysin-3 (Str3) and gelatinase A, the serine proteinases urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), and the cysteine proteinase cathepsin B (Ossowski et al., 1979; Busso et al., 1989; Dickson and Warburton, 1992; Lefebvre et al., 1992; Strange et al., 1992; Talhouk et al., 1992; Guenette et al., 1994; Li et al., 1994; Lund et al., 1996). The spatial distribution of mRNAs for Str1, Str3, gelatinase A and uPA in fibroblast-like cells during involution (Lefebvre et al., 1992; Lund et al., 1996) points to an active role for the mesenchymal stroma during tissue remodeling.

In mice that overexpress an autoactivating Str1 transgene during pregnancy and in cultured mammary epithelial cells, the degradation of extracellular matrix (ECM) appears to initiate apoptosis (Boudreau et al., 1995; Alexander et al., 1996). Str1 also participates in the degradation of the basement membrane macromolecule entactin/nidogen-1. This degradation is attenuated in double transgenic mice that overexpress both Str1 and tissue inhibitor of matrix metalloproteinases-1, leading to a decrease in the overall proteolytic potential (Alexander et al., 1996). These experiments directly demonstrate a functional role for Str1 during mammary epithelial apoptosis (Boudreau et al., 1995; Alexander et al., 1996).

Plasmin produced from plasminogen (Plg) by uPA and tPA may also influence mammary gland development through activation of latent pro-MMPs, direct degradation of ECM substrates, or the release of bioactive ECM fragments and growth factors (see Sternlicht and Werb, 1999, for review). To explore the molecular and cellular mechanisms underlying the role of plasmin(ogen) in these tissue remodeling events, we have analyzed mammary gland morphology in the $Plg^{-/-}$ mouse. We used stereological methods to obtain unbiased quantitative estimates of the structural composition and contents of the mammary gland, and biochemical and molecular analyses to determine the molecular mechanisms underlying the morphological changes.

MATERIALS AND METHODS

Animals and tissue treatment

Plg gene-targeted 129/Black Swiss mice (Bugge et al., 1995) were backcrossed into C57BL/6J (Panum Institute, Copenhagen) for 4 or 10 generations, and 8- to 12-week-old female mice were examined. All mice backcrossed for four generations appeared healthy and were of the same mass as $Plg^{+/+}$ controls until after 14 weeks of age. *uPA* gene-targeted 129 mice (Carmeliet et al., 1994) were backcrossed into C57Bl/6J for four generations. To normalize lactational pressure, the number of pups for each dam was adjusted to seven on post-partum day 1. Pups were removed after 10 days of lactation and mammary glands were collected immediately or after 5 days of involution. The mice were anesthetized by subcutaneous injection of 0.03 ml/10 g of a 1:1 mixture of Dormicum (Midazolam, 5 mg/ml) and Hypnorm (Fluanison, 5 mg/ml and Fentanyl, 0.1 mg/ml). The mice were perfused intracardially with 20 ml of ice-cold phosphate-buffered saline (PBS) and inguinal and thoracic mammary glands were removed for protein extraction as described below. Except where otherwise stated, all lactating and post-lactational mice studied were primiparous. Animal care at the University of Copenhagen and Copenhagen University Hospital, Copenhagen, Denmark was in accordance with national and institutional guidelines and all mice were found to be free of murine pathogens in accordance with the FELASSA recommendations for health monitoring of experimental units (Rehbinder et al., 1996).

Mice used for immunohistochemistry were treated similarly, except that perfusion with cold PBS was followed by intracardial perfusion/fixation with 20 ml 4% (w/v) paraformaldehyde (PFA) in PBS. Abdominal (#4) mammary glands were removed, weighed and fixed for 16 hours in 4% PFA. The tissue was then rinsed in PBS, dehydrated and embedded in paraffin. Tissue sections were floated onto SuperFrost$^+$ slides (Fisher Scientific, Pittsburgh, PA, USA) and stained with Hematoxylin and Eosin, and Gomori's one-step trichrome stains. Apoptotic cells were identified using an in situ apoptosis detection kit (Boehringer-Mannheim, Penzberg, Germany). After counterstaining, at least 1000 nuclei per specimen were counted. Whole-mount analysis of abdominal mammary glands was performed as previously described (Sympson et al., 1994).

Stereological analysis

Abdominal mammary glands were systematically sectioned at equidistant levels 20-200 μm apart and perpendicular to the long axis, depending on the size of the gland. At each level, 4-5 μm sections were cut and stained as described above. To obtain unbiased sampling, the first sections from at least three levels (top, middle and bottom) of each gland were analyzed using standard stereological methods (Gundersen et al., 1988). Morphometric measurements were carried out on coded slides by an observer (S.F.B.) who was unaware of the mouse category. Area fractions were estimated by point counting with a microscope that projected a 491× brightfield image together with a grid onto a computer screen. The grid had several sets of points with appropriate ratios (1:2, 1:4, 1:8, 1:18). 'Fine' points 92 μm apart were used for low-volume structures and 'coarse' points for high-volume structures and the total tissue reference volume. The specimen stage was moved in a meandering pattern by a DC-motor to ensure that the mammary tissue and grid were positioned independently. Using this procedure, about 10% of the total area of each section was evaluated. The overall mammary volume that was made up of a given structure (e.g. alveoli) was estimated as the product of its area fraction and the wet mass (mg) of the corresponding mammary gland, expressed as mm^3, assuming a glandular density of 1 mg/mm^3.

Statistical analysis

Mammary gland wet masses were compared using one-way analysis of variance (ANOVA). Comparisons of morphometric data for all three genotypes were obtained using the Kruskall-Wallis test. Approximate 95% confidence intervals were estimated for the ratios of mean involuting to lactating mammary gland wet masses. Tests on proportions (e.g. the fraction of mice that failed to feed their pups) were done using exact methods. *P*-values less than 0.05 were considered significant. All calculations were performed using the SAS System version 6.12 (SAS Institute, Cary, North Carolina, USA).

Immunohistochemical analysis

Tissue sections were deparaffinized in xylene and hydrated through graded ethanol/water dilutions. Antigen retrieval was by proteolytic digestion with 0.025% trypsin (Sigma T8128) in 50 mM Tris (pH 7.6) containing 0.1% $CaCl_2$ for 5-6 minutes at 37°C. Endogenous peroxidase activity was blocked using 1% hydrogen peroxide for 15 minutes at ambient temperature. Sections were then washed in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.6) containing 0.5% Triton X-100 (TBS-T). The slides were then mounted into Shandon racks with immunostaining coverplates (AX-LAB, Copenhagen, Denmark) for subsequent incubations. Rabbit anti-mouse fibrin IgG (10 mg/ml; Bugge et al., 1995) were incubated at 1:1000, a biotinylated rat monoclonal antibody against activated mouse macrophages (clone BM-8; BMA Biomedicals, Augst, Switzerland; Malorny et al., 1986) was incubated at 1:30. Primary antibodies were incubated overnight at 4°C. Rabbit anti-fibrin IgG were detected with biotinylated, affinity-purified swine anti-rabbit IgG (DAKO, Glostrop, Denmark) followed by streptavidin-HRP complexes (DAKO). Biotinylated BM-8 was detected with streptavidin-HRP complexes followed by tyramide signal amplification. All antibody incubations were followed by washes with TBS-T. Sections were developed with 0.25 mg/ml 3-amino-9-ethylcarbazole (AEC) in 0.05 M buffered acetic acid (pH 5.0) for 10 minutes, counterstained in Mayer's Hematoxylin for 30 seconds, and mounted in Glycergel (DAKO).

ELISA for uPA

Preparation of mammary gland extracts for uPA ELISA was as described (Lund et al., 1996). Nunc 96-well immunoplates were coated overnight with 4 μg/ml MA-H77A10 (directed against mouse

uPA) in 0.1 M Na_2CO_3, pH 9.8, at 4°C. The wells were then washed with PBS-T and remaining protein-binding sites were blocked with 1% BSA in PBS at 37°C. Samples and a mouse uPA standard (concentration determined by amino acid analysis) were diluted in dilution buffer (pH 7.4) containing 1% BSA and 0.1% Tween-20, and incubated for 1 hour at 37°C. Bound uPA was detected using a polyclonal rabbit anti-mouse uPA IgG diluted to 2 μg/ml in dilution buffer. The signal was measured as enzyme rate (kinetic ELISA) using an alkaline phosphatase-conjugated monoclonal antibody against rabbit IgG (Sigma).

Zymography and western blot analysis

Pieces of mammary gland were homogenized in RIPA lysis buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) at 0.25 mg wet mass/μl and homogenates were spun at 10,000 ***g*** for 15 minutes at 4°C. Soluble fractions were diluted in nonreducing SDS sample buffer for zymography (Herron et al., 1986), and insoluble fractions were washed once in RIPA buffer and boiled in reducing SDS sample buffer for western blot analysis. To visualize gelatinolytic enzymes, RIPA-soluble proteins were separated on 10% SDS-PAGE mini-gels containing 1 mg/ml gelatin. After electrophoresis, the gels were washed for 30 minutes at room temperature in renaturing buffer (10 mM Tris-HCl, pH 7.5, 2.5% Triton X-100), incubated for 24-48 hours at 37°C in enzyme buffer (50 mM Tris-HCl, pH 7.6, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij-35), and stained with Coomassie Brilliant Blue R-250. Gelatinolytic enzymes were thus revealed after destaining as clear bands against a background of uniformly stained substrate. To visualize entactin and its degradation products, reduced RIPA-insoluble proteins were resolved on 8% SDS-PAGE mini-gels and transferred to nitrocellulose membranes by electrophoretic blotting. Membranes were blocked for 2 hours at room temperature with 5% BSA in TBS containing 0.5% Tween-20 and 0.1% Triton X-100 (TBSTT, pH 7.6), and incubated overnight at 4°C with 0.2 μg/ml rat anti-mouse entactin (Upstate Biotechnology, Inc. #05-208) in TBSTT containing 0.5% BSA. After washing in TBSTT, membranes were incubated for 2 hours at room temperature with species-specific, peroxidase-conjugated sheep anti-rat IgG (Amersham #NA9320) diluted 1:2000 in TBSTT containing 0.5% BSA. Peroxidase activity on washed blots was detected using enhanced chemiluminescence (ECL) reagents from Amersham.

RESULTS

Plasminogen deficiency compromises fertility and lactational competence

By definition, the study of lactational competence in mutant mice requires that they successfully reproduce. We found that 26% of $Plg^{-/-}$ female mice (n=32) on a mixed 129/Swiss Black genetic background backcrossed for four generations onto C57BL/6J failed to give birth, whereas only 6% of wild-type littermates (n=30) and 10% of $Plg^{+/-}$ mice (n=27) failed to do so (P<0.04). Even though $Plg^{-/-}$ mice had apparently normal estrus cycles (as judged by vaginal smears) and had been caged with two separate male mice of proven fertility for 2 weeks each, remnants of embryos and implantation sites were uniformly absent in those $Plg^{-/-}$ mice that failed to bear pups. This indicates that the mice had failed to become pregnant and suggests that Plg deficiency had compromised their fertility. When the mutant mice were further backcrossed to C57BL/6J for 10 generations, this phenotype became more severe. Fewer than 50% of the C57BL/6J $Plg^{-/-}$ mice became pregnant. For this reason, lactational studies were performed using the mixed background that had been backcrossed to C57BL/6J for four generations, except where noted. Although a significantly lower proportion of $Plg^{-/-}$ mice became pregnant than did $Plg^{+/-}$ or $Plg^{+/+}$ mice, all groups produced the same size litters (n≈8). Second pregnancies were rare in $Plg^{-/-}$ mice of all backgrounds.

We next examined lactational competence in primiparous $Plg^{-/-}$ mice. To normalize the lactational pressure for all mice, the number of pups was adjusted to 7 on post-partum day 1. Wild-type and $Plg^{+/-}$ mice rarely (7%) failed to lactate, whereas 28% of $Plg^{-/-}$ mice backcrossed for four generations into C57BL/6J and >75% of $Plg^{-/-}$ mice backcrossed for ten generations into the C57BL/6J background were unable to sustain lactation for 10 days (data not shown). The dams showed normal mothering behavior and, in most cases, some milk was initially seen in the stomachs of pups. Even so, the pups failed to thrive rapidly thereafter. This lactational failure was even more severe after a second pregnancy. Out of eight second pregnancies observed in the mixed background, only one litter survived without loss.

Although most of the lactating $Plg^{-/-}$ mice produced enough milk to sustain 7 pups for 10 days, their mammary glands were histologically abnormal when compared to those of wild-type mice (Fig. 1A-D). After 10 days of lactation, the mammary glands of $Plg^{-/-}$ and $Plg^{+/-}$ mice were lighter and contained less total secretory alveolar tissue than the glands of their $Plg^{+/+}$ littermates (Fig. 1G,H). The mean mammary gland wet masses for each genotype were significantly different (P<0.0001, ANOVA), with the $Plg^{+/-}$ being higher than $Plg^{-/-}$ (P<0.01) and lower than $Plg^{+/+}$ (P<0.01). The hypothesis of equal total alveolar volumes was rejected (P<0.002, Kruskall-Wallis test), $Plg^{+/-}$ being significantly lower than $Plg^{+/+}$ (P<0.003), whereas there was no significant difference between $Plg^{+/-}$ and $Plg^{-/-}$. $Plg^{+/-}$ mice exhibited an intermediate phenotype, suggesting haploinsufficiency. Not surprisingly, we also observed histological abnormalities in mice that had failed to lactate. The mammary glands from 7 of the 9 $Plg^{-/-}$ dams that had been unable to nurse their pups contained almost no secretory alveolar epithelium when examined immediately after the death of their litters. Instead, they had regions of prominent fibrosis, scattered areas of atypical (multilocular, immature) adipose tissue, and, in two cases, abundant inflammatory cells (Fig. 1E,F). The mammary glands from the two $Plg^{+/+}$ and two $Plg^{+/-}$ mice that failed to lactate for 10 days also exhibited minimal alveolar development, but in an adipocyte-rich stroma without fibrosis.

Fibrin did not accumulate in vascular or extravascular tissues of lactating mammary glands in $Plg^{-/-}$ mice (Fig. 2A,B). The distribution of immunoreactive α-smooth muscle actin in myoepithelial and vascular smooth muscle cells was unchanged in $Plg^{-/-}$ and $Plg^{+/-}$ mice (data not shown). However, numerous activated macrophages were found in the stroma of lactating $Plg^{-/-}$ glands by staining with a monoclonal antibody (BM-8), whereas there were few immunoreactive cells in wild-type mammary glands (Fig. 2C,D).

Plasminogen is required for normal mammary gland involution

The expression of uPA is highly upregulated during post-lactational mammary gland involution (Lund et al., 1996), thus its physiological target, Plg, may play a critical role in involution. After 10 days of successful lactation and 5 days of

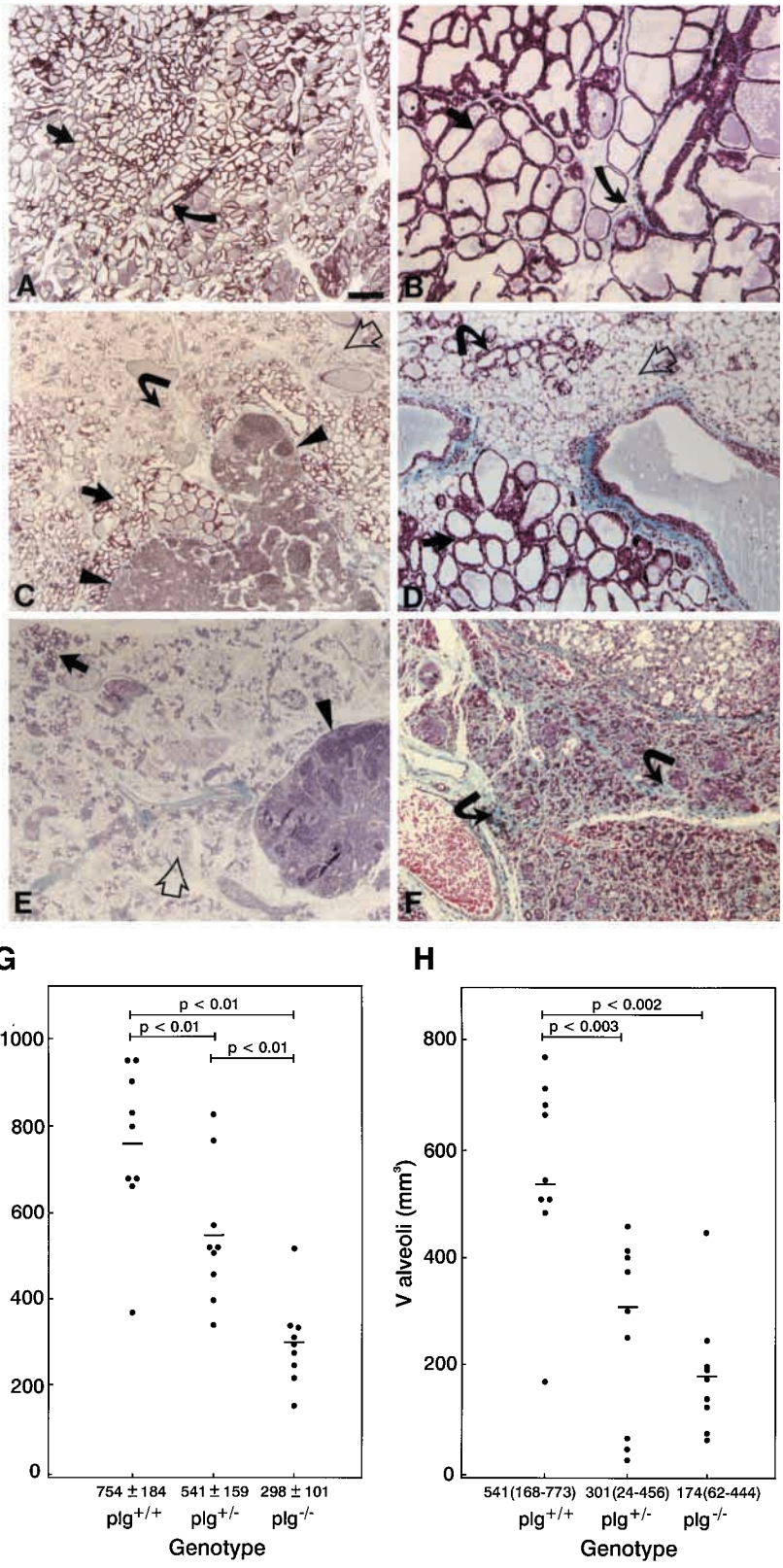

Fig. 1. Trichrome-stained tissue sections, wet masses and alveolar volumes for mammary glands from $Plg^{+/+}$, $Plg^{+/-}$ and $Plg^{-/-}$ dams 10 days after parturition. (A,B) Mammary gland from a lactating $Plg^{+/+}$ mouse. Note the closely packed, distended secretory alveoli (straight arrows) and sparse periductal connective tissue (curved arrows) with minimal blue-stained collagen. (C,D) Mammary gland from a lactating $Plg^{-/-}$ mouse. Although some areas contain well-developed secretory alveoli (closed straight arrows), others contain enlarged (ectatic) ducts, undeveloped alveoli (curved arrows) and abundant adipose tissue (open arrows). (E,F) Mammary gland from a $Plg^{-/-}$ mouse that failed to lactate for 10 days. Few secretory alveoli (straight arrow) are present, whereas adipose (open arrow) and connective tissues (curved arrows) are abundant. Arrowheads in C and E indicate mammary lymph nodes. Bar, 400 μm (A,C,E) and 100 μm (B,D,F). (G) Wet masses of individual mammary glands for each genotype. *P* values indicate that the mean glandular wet masses (horizontal bars and values ± s.d. given along the lower axis) were significantly different for each group. (H) Total alveolar volumes (V alveoli) for individual mammary glands of each genotype. Median alveolar volumes (horizontal bars and values given with ranges along the lower axis) for $Plg^{+/-}$ and $Plg^{-/-}$ glands were significantly smaller than those of $Plg^{+/+}$ glands.

involution, the median glandular wet masses for $Plg^{+/+}$, $Plg^{+/-}$ and $Plg^{-/-}$ dams were significantly different from one another ($P<0.0001$, ANOVA) with the mammary glands of $Plg^{+/-}$ mice weighing less than those of $Plg^{-/-}$ mice ($P<0.05$) and more than those of $Plg^{+/+}$ mice ($P<0.01$) (Fig. 3G). Thus, although the mammary glands from lactating $Plg^{-/-}$ mice were initially smaller than those of their $Plg^{+/-}$ and $Plg^{+/+}$ littermates (Fig. 1G), they ended up significantly larger than those of their heterozygous and wild-type littermates after 5 days of involution. As a result, the proportionate reduction in the median gland mass after 5 days of involution was dramatically different for the three genotypes; namely an 86% reduction in mammary gland size in wild-type mice, a 67% reduction in heterozygous mice, and only a 27% reduction in $Plg^{-/-}$ mice.

The differences in mass of the involuting mammary glands were accompanied by profound histological differences (Fig. 3A-I). The alveolar tissue in $Plg^{-/-}$ mice revealed only minimal regression (Fig. 3K). Residual alveolar and ductal structures each occupied significantly larger volume fractions in $Plg^{-/-}$ and $Plg^{+/-}$ animals than in wild-type animals. Collapsed alveoli with epithelial cells shed into the ducts and uncollapsed necrotic alveoli were seen in 7 of the 13 $Plg^{-/-}$ glands, but were never seen in $Plg^{+/+}$ or $Plg^{+/-}$ glands. In addition, total volumes for alveolar, ductal and combined connective and vascular tissue compartments were each significantly larger in $Plg^{-/-}$ and $Plg^{+/-}$ glands than in $Plg^{+/+}$ glands, with heterozygous mice exhibiting intermediate values that were generally closer to those seen in $Plg^{-/-}$ mice than

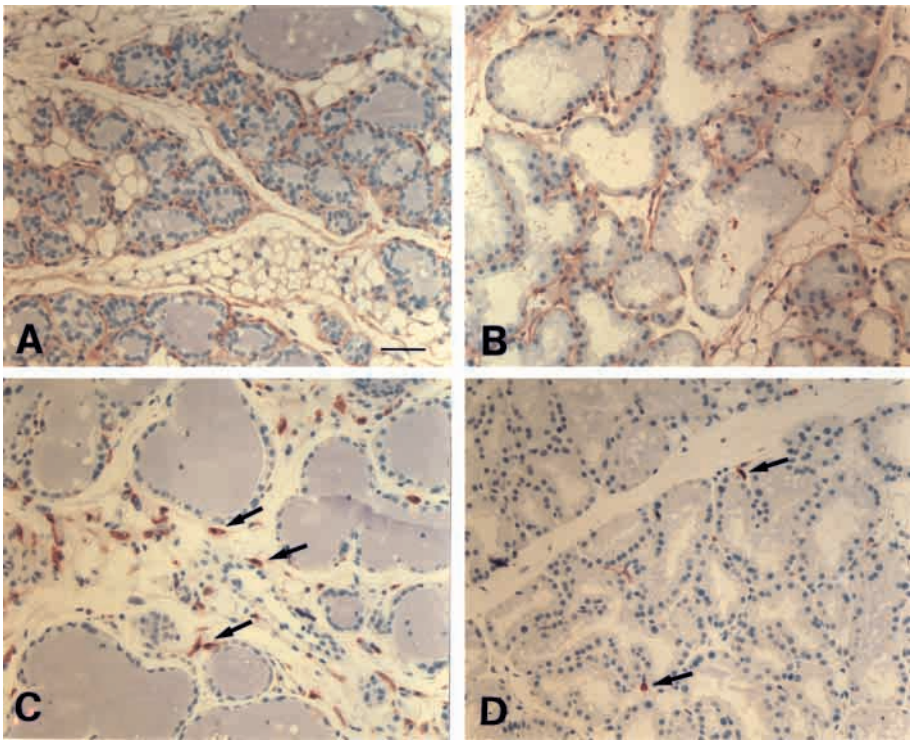

Fig. 2. Immunoperoxidase staining of lactating mouse mammary glands for fibrin, and macrophages. Sections of glands from $Plg^{-/-}$ (A,C) and $Plg^{+/+}$ (B,D) mice were incubated with polyclonal antibodies to mouse fibrin(ogen) (A,B), and a macrophage-specific monoclonal antibody (C,D). The fibrin(ogen)-specific antibodies recognized vascular and extravascular fibrin(ogen), and the macrophage-specific antibody revealed the presence of numerous macrophages in the stroma of $Plg^{-/-}$ mammary glands (arrows in C), but only a few macrophages in $Plg^{+/+}$ mammary tissue (arrows in D). Bar, 50 μm.

wild-type mice. Thus, only 1 mm^3 of alveolar tissue remained in $Plg^{+/+}$ mammary glands following 5 days of involution, whereas 8.6 and 8.9 mm^3 of residual alveolar tissue persisted in $Plg^{+/-}$ and $Plg^{-/-}$ glands, respectively. These differences are even more remarkable when one considers that 5 days earlier, when pups were removed from their nursing dams, the total alveolar volume was significantly smaller in lactating $Plg^{+/-}$ and $Plg^{-/-}$ glands than in wild-type glands. Thus, the median alveolar volume was reduced 306-fold (95% confidence interval=140-471) over 5 days of involution in $Plg^{+/+}$ glands, but only 32-fold (13-50) and 11-fold (3-20) in $Plg^{+/-}$ and $Plg^{-/-}$ glands, respectively.

Fibrin deposition is not enhanced in mammary glands of plasminogen-deficient mice

The data presented above indicate that mammary involution is abnormal in $Plg^{-/-}$ mice. This raises the question of the mechanisms responsible for this failure in mammary gland remodeling. The inability to efficiently degrade fibrin, which acts as a provisional matrix, accounts for the impaired wound healing phenotype in $Plg^{-/-}$ mice (Bugge et al., 1996; Rømer et al., 1996; Lund et al., 1999). Mammary epithelial cells can use fibrin gels as an alternative to ECM for growth in culture (Alford et al., 1998). Thus, if excess fibrin accumulates in $Plg^{-/-}$ mice, it could act as a provisional matrix for mammary epithelial cells, supporting their prolonged survival and function in the face of ECM degradation and other pro-apoptotic signals seen during involution. Nevertheless, we did not observe excess fibrin accumulation during involution in the $Plg^{-/-}$ mice compared to $Plg^{+/+}$. Wild-type and $Plg^{-/-}$ involuting mammary glands showed similar immunohistochemical staining for intra- and extravascular fibrin (Fig. 4A,B). In lactating $Plg^{-/-}$ glands the lack of abnormal fibrin deposition may be due to fibrin clearance by increased macrophage infiltration (see Fig. 2C,D) via plasmin-independent mechanisms (Simon et al., 1993; Hiraoka et al., 1998). There was, however, no similar increase in the number of activated macrophages in involuting $Plg^{-/-}$ glands as compared to wild-type glands (Fig. 4C,D).

Stromal differentiation is altered in involuting plasminogen-deficient mammary glands

Important paracrine crosstalk occurs between mammary epithelial cells and the adipocyte-rich stroma of the mammary gland. Indeed, a reciprocal relationship exists between adipocyte and epithelial differentiation, with one population increasing as the other wanes during pregnancy and involution (Topper and Freeman, 1980). In $Plg^{-/-}$ mice, delayed epithelial regression was accompanied by diminished adipocyte differentiation during involution. The $Plg^{-/-}$ glands had proportionally less adipose tissue than wild-type or $Plg^{+/-}$ glands, and their stroma was instead filled with immature adipocytes and fibrotic tissue (Fig. 3A-I). The fraction of mammary tissue containing adipocytes was also statistically different for each genotype (P=0.0002; Kruskal-Wallis test) at 50% for $Plg^{+/+}$, 46% for $Plg^{+/-}$ and 34% for $Plg^{-/-}$ mice. These data suggest that an abnormal stromal environment may contribute to the altered epithelial phenotype.

uPA and gelatinase B are increased in lactating plasminogen-deficient mice

uPA and matrix-degrading MMPs are highly regulated during mammary gland development (Busso et al., 1989; Lund et al., 1996). We next asked whether Plg deficiency alters their expression or enzymatic activation, because such alterations could clearly contribute to the mammary changes seen in

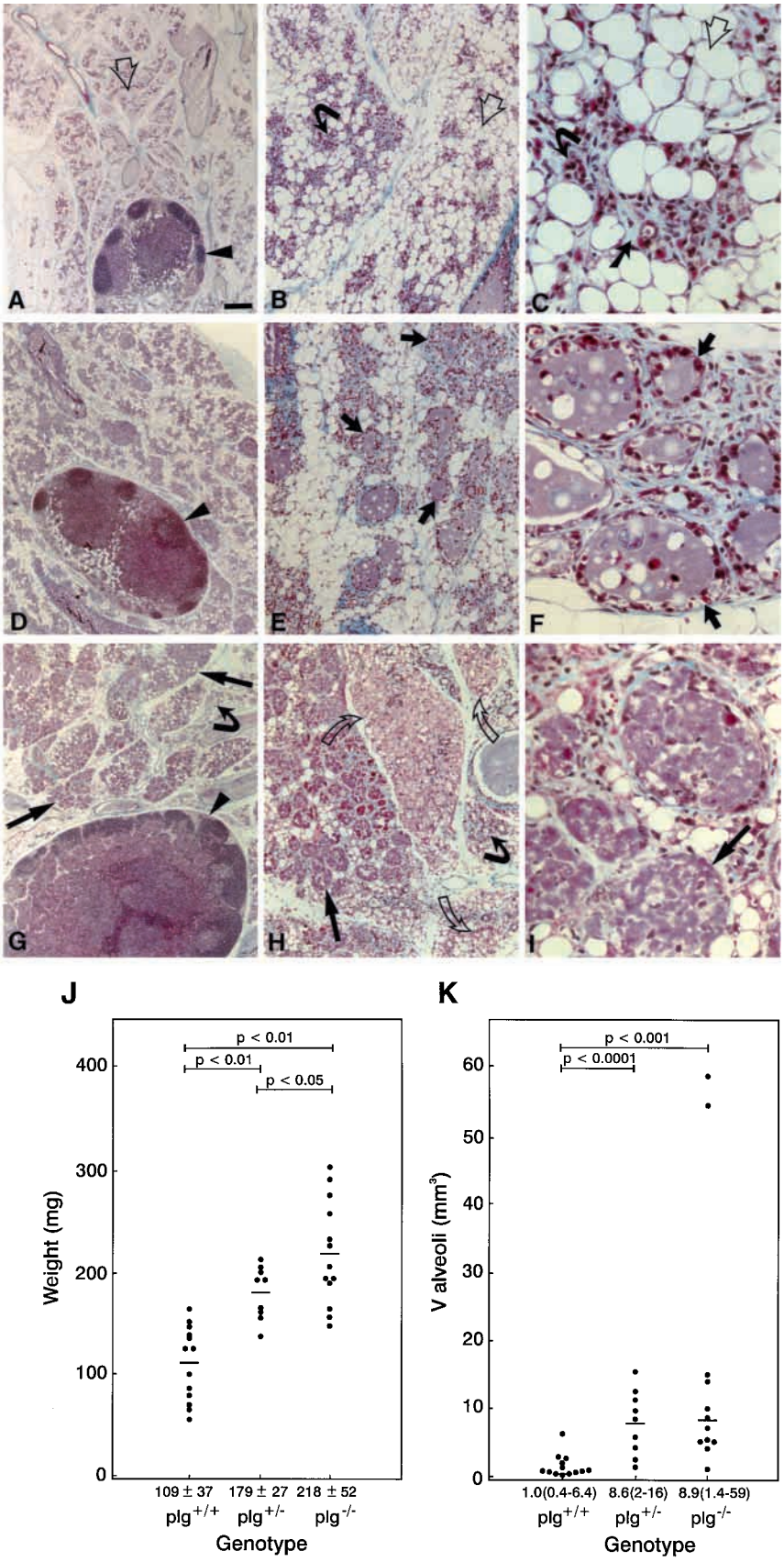

Fig. 3. Trichrome-stained tissue sections, wet masses and alveolar volumes for mammary glands from $Plg^{+/+}$, $Plg^{+/-}$ and $Plg^{-/-}$ mice after 5 days of involution. (A-C) Involuting gland from a $Plg^{+/+}$ mouse. The alveoli (B,C, curved arrows) have regressed and are surrounded by connective tissue (C, straight closed arrow) and extensive adipose tissue (open arrows). (D-F) Involuting gland from a $Plg^{+/-}$ mouse. Non-collapsed alveoli and ducts are seen in several areas (arrows), indicating incomplete regression of the secretory apparatus. (G-I) Involuting gland from a $Plg^{-/-}$ mouse. Alveolar regression is incomplete and areas of residual non-collapsed alveoli containing proteinaceous material are present (straight arrows). The adipose tissue in some areas has an abnormal multilocular appearance (open curved arrow in H). Resident and infiltrating cells are also present within the stroma (closed curved arrows in G,H) and intra-mammary lymph nodes are indicated (arrowheads in A,D,G). Bar, 400 μm (A,D,G), 100 μm (B,D,F) and 25 μm (C,F,I). (J) Wet masses of individual post-lactational mammary glands after 5 days of involution. The mean glandular wet masses (horizontal bars and values ± s.d. given along the lower axis) were significantly different for each group. (K) Total alveolar volumes (V alveoli) for individual mammary glands of each genotype. Median alveolar volumes (horizontal bars and values given with ranges along the lower axis) for $Plg^{+/-}$ and $Plg^{-/-}$ glands were significantly larger than those of $Plg^{+/+}$ glands.

lactating and involuting $Plg^{-/-}$ mice. As seen previously (Lund et al., 1996), uPA levels were low during lactation and were upregulated five- to sixfold during involution in $Plg^{+/+}$ mice; similar levels were found in $Plg^{+/-}$ mice (Fig. 5A). In $Plg^{-/-}$ mice, however, mammary uPA levels were fourfold higher than in wild-type mice during lactation (P<0.02). Only a slight increase of this already high concentration of uPA was seen during involution, so that uPA levels in involuting $Plg^{-/-}$ mammary glands were comparable to those seen in wild-type mice. Because mammary changes were observed during lactation, when uPA is normally low, they may arise during prior stages of mammary development when uPA is more abundant and Plg activation is more apt to have an impact. However, mammary glands from $uPA^{-/-}$ mice showed no detectable delay in development or postlactational involution by whole-mount or histological analysis (data not shown). These data suggest that uPA is not essential and may serve as a marker of the macrophage infiltrate (Fig. 2C,D).

There were no pronounced differences between the three genotypes in the expression of gelatinase A during either lactation or involution, although its activation during involution appeared to be slightly lower in $Plg^{-/-}$ glands as compared to $Plg^{+/-}$ and $Plg^{+/+}$ glands (Fig. 5B,C).

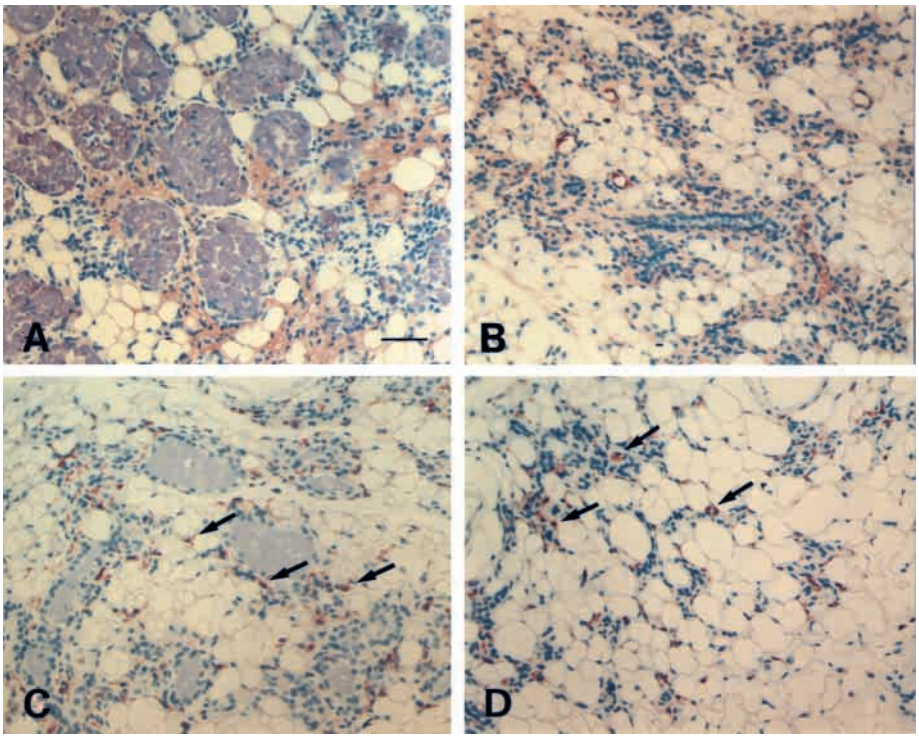

Fig. 4. Immunoperoxidase staining of involuting mouse mammary glands for fibrin and macrophages. Sections of glands from $Plg^{-/-}$ (A,C) and $Plg^{+/+}$ (B,D) mice 5 days after weaning were stained with polyclonal antibodies to mouse fibrin(ogen) (A,B) and a macrophage-specific monoclonal antibody, BM-8 (C,D). The fibrin(ogen)-specific antibodies recognized both vascular and extravascular fibrin(ogen) immunoreactivity in both $Plg^{+/+}$ and $Plg^{-/-}$ involuting mammary tissue (red color in A,B). The BM-8 monoclonal antibody identified numerous macrophages (arrows in C,D) in the stroma of both $Plg^{-/-}$ and $Plg^{+/+}$ involuting mammary tissue. Note that the glands of involuting $Plg^{-/-}$ mice (A,C) retain the histological features of lactating glands (Fig. 2). Bar, 50 μm.

In the $Plg^{+/+}$ and $Plg^{+/-}$ mice, gelatinase B levels were low in the lactating glands and were consistently higher on the fifth day of involution. Both latent and active gelatinase B levels were unusually high in most $Plg^{-/-}$ mammary glands during lactation (Fig. 5B), but were similar to those seen in the $Plg^{+/+}$ and $Plg^{+/-}$ mice during involution (Fig. 5C). Because uPA and gelatinase B are produced by macrophages, their unusually high expression may be attributed to the unusual abundance of activated, BM-8-immunoreactive macrophages that were also present in the mammary glands of lactating (Fig. 2C,D), but not involuting (Fig. 4C,D), $Plg^{-/-}$ mice. The high levels of gelatinase B may compensate for the effect of Plg-deficiency (e.g. by preventing fibrin accumulation during lactation; Hiraoka et al., 1998). It is noteworthy that both gelatinase A and B continued to be activated in vivo despite the absence of Plg. A 75 kDa gelatinolytic activity was detected in lactating, but not involuting, glands from $Plg^{+/+}$, $Plg^{+/-}$ and $Plg^{-/-}$ mice (Fig. 5B,C). Inhibition experiments showed that the activity was completely abolished by aprotinin, but not by EDTA, calpain I and II, and E64, suggesting that the activity was probably caused by a serine protease (data not shown).

ECM degradation is altered during mammary gland involution in plasminogen-deficient mice

Our data indicate that the stromal ECM is abnormal in the $Plg^{-/-}$ glands, showing an increase in collagen deposition (Fig. 3). However, MMP expression and activation were not substantially altered in $Plg^{-/-}$ mice. Therefore, we sought to determine if basement membrane remodeling was altered. Following weaning, a characteristic pattern of proteolysis of various components of the extracellular matrix is seen (Alexander et al., 1996). We found that the ECM component entactin/nidogen-1 was degraded in $Plg^{+/+}$, $Plg^{+/-}$ and $Plg^{-/-}$ glands. By immunoblotting analysis, the level of 112 and 110 kDa degradation products of entactin were virtually the same in the three genotypes both at day 10 of lactation and at day 5 of involution (data not shown). However, bands at about 58 and 55 kDa, that were weakly detectable in wild-type mice, were strongly increased in $Plg^{+/-}$ and $Plg^{-/-}$ mice during both lactation (data not shown) and involution (Fig. 5D), suggesting that plasmin is required to prevent accumulation of lower molecular weight degradation products. We found that laminins 1 and 5 were degraded in involuting mammary glands; however, we observed no differences in the degradation patterns for the β1 or γ1 chain of laminin-1 or the γ2 chain of laminin-5 for the various Plg genotypes (data not shown). These results indicate that although ECM degradation does take place, there is an accumulation of specific basement membrane protein fragments that probably require plasmin for further degradation. Taken together with the accumulation of a fibrotic stromal matrix, they indicate that mammary ECM homeostasis is severely compromised in $Plg^{-/-}$ mice. Moreover, such fragments of ECM could have new biological activities in involution (Pujuguet et al., 2000).

Because ECM remodeling is coupled to epithelial apoptosis during involution (Boudreau et al., 1995), one consequence of altered ECM homeostasis might be altered apoptosis. Indeed, we observed decreased apoptosis in $Plg^{-/-}$ mammary glands at 5 days of involution. Apoptotic cells were detected in mammary glands of each genotype at 5 days post-weaning (Fig. 6A,B); however, the proportion of epithelial cells undergoing apoptosis in $Plg^{-/-}$ glands (2.0±0.5%; n=3) was significantly lower than in $Plg^{+/+}$ glands (5.2±0.5%; n=3, P<0.001). These data suggest that alveolar regression is delayed, but not entirely blocked. Indeed, histological analysis of mammary glands from $Plg^{-/-}$ mice 7 and 14 days post-weaning showed continued but incompleted remodeling

compared to wild-type glands (Fig. 6C-F). Interestingly, mammary glands harvested from a $Plg^{-/-}$ mouse 21 days after its second parturition and unsuccessful lactation had not involuted and were still engorged with milk (data not shown). Thus the delay in alveolar regression was even more severe after a second pregnancy.

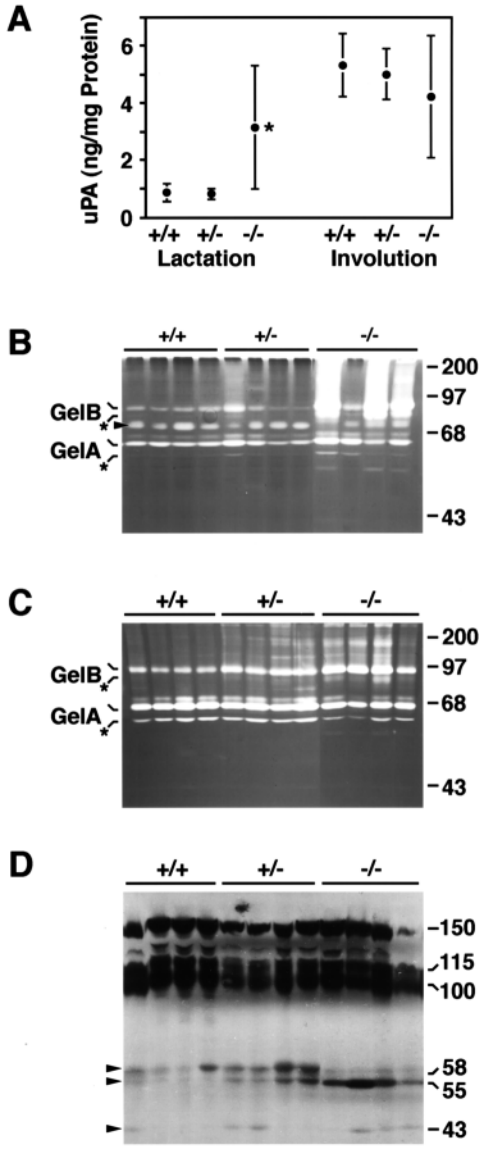

Fig. 5. Biochemical analysis of matrix-degrading enzymes and ECM components in lactating and involuting mammary glands. (A) The concentration of uPA was determined by ELISA and protein content was determined using Folin-Ciocalteu reagent. Values shown are means ± s.d. *P<0.02 versus lactating $Plg^{+/+}$ glands.
(B,C) Zymograms for gelatinases A and B (GelA and GelB) in lactating and involuting mammary glands. Representative mammary extracts from four different mice of each genotype are shown for lactating (B) and involuting (C) mice. Activated forms of GelA and GelB are distinguished from the latent (propeptide-containing) gelatinases by asterisks. A 75 kDa gelatinolytic serine proteinase (arrowhead in B) appeared to be unaltered by Plg deficiency.
(D) Protein blot analysis for entactin from involuting mammary glands. Low molecular weight entactin degradation products (arrowheads) showed differential, genotype-specific accumulation, whereas 110 and 115 kDa breakdown products showed similar abundance for each genotype.

DISCUSSION

Plasminogen is required for normal mammary gland development

The tight regulation of uPA expression during mammary gland development (Busso et al., 1989; Lund et al., 1996) suggests that its physiological target, Plg, may play an important role in these processes. By examining $Plg^{-/-}$ mice, we have shown that Plg is indeed required for adequate lactation and post-lactational involution. Moreover, a haploinsufficiency phenotype was observed in $Plg^{+/-}$ mice, suggesting that these processes require a threshold level of Plg that is not achieved by one allele alone. In accordance with indications that Plg may play a permissive role in ovulation (Hägglund et al., 1996) and embryonic implantation (Sappino et al., 1989), we also observed diminished fertility in $Plg^{-/-}$ female mice. In addition, it is likely that modifiers of Plg activity exist, based on our observations of strain-dependent differences in phenotypic severity.

Plg does not appear to be required during lactation, when uPA expression is low (Busso et al., 1989), but prior to lactation, when uPA expression is high and the lactational phenotype is acquired. Indeed, between 28% and more than 75% of the primiparous $Plg^{-/-}$ mice were unable to lactate successfully, depending on the strain. In addition, the mammary glands of those $Plg^{-/-}$ mice that were able to lactate were significantly smaller and had significantly smaller secretory alveolar volumes than those of their $Plg^{+/+}$ and $Plg^{+/-}$ littermates. The $Plg^{-/-}$ dependent defects seen in lactation probably arose during mammary development. Indeed, whole-mount preparations from 6- to 8-week-old virgin $Plg^{-/-}$ mice in estrus revealed delayed ductal branching during development, even though they appeared to initiate puberty at the same time as $Plg^{+/+}$ mice, cycled normally, and were healthy and of normal body mass (our unpublished observations). These data demonstrate that Plg plays a significant role in achieving lactational competence during pregnancy. In support of the notion that lactation itself does not require Plg, the existing alveolar epithelium required for milk production and the myoepithelial cells required for milk ejection remained functional in $Plg^{-/-}$ mice, so that some were able to nurse their pups despite a severe shortage of secretory epithelium.

Lactational failure in the absence of sufficient Plg could also reflect inadequate mammary development during puberty, when uPA levels are high (Busso et al., 1989). Indeed, whole-mount analysis of mammary glands from virgin $Plg^{-/-}$ mice showed a delay in early ductal development (data not shown). Thus, Plg may play an important role in branching morphogenesis, which, if lacking, could limit lactational competence. Lactational failure was even more prevalent following the rare occurance of a second pregnancy. Thus, although $Plg^{-/-}$ mammary glands may involute to some extent, their phenotype becomes progressively more severe due to an inability to dedifferentiate and redifferentiate to a normal lactating state. These data also suggest that abnormal

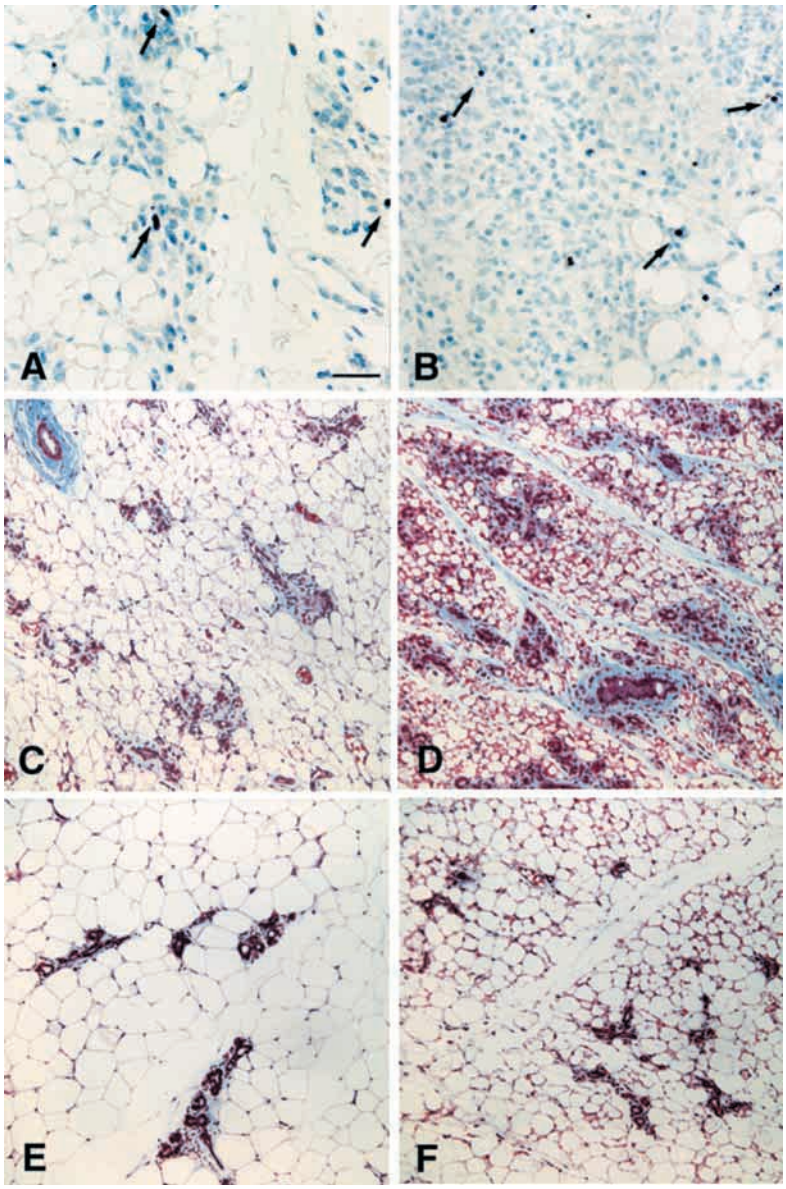

Fig. 6. TUNEL staining for apoptotic cells and trichrome-staining of involuting mammary glands from $Plg^{+/+}$ (A,C,E) and $Plg^{-/-}$ (B,D,F) mice. Arrows indicate apoptotic cells in mammary glands isolated 5 days after weaning (A,B). Trichrome staining of mammary glands isolated 7 days (C,D) and 14 days (E,F) after weaning. Bar, 50 μm.

or incomplete involution can compromise lactational development during a subsequent pregnancy.

Mammary gland involution in mice occurs in two distinct phases: an initial wave of apoptosis that begins in the absence of high proteinase expression 2 days after weaning, and a proteinase-dependent phase of glandular regression and ECM remodeling that starts 2 days later (Lund et al., 1996). This latter phase is associated with strong upregulation of uPA and MMPs (Lund et al., 1996) and can be suppressed by administering inhibitors of both uPA and MMPs, but not by administering either inhibitor alone (unpublished results). Nevertheless, our results indicate that post-lactational involution is profoundly compromised in the absence of Plg. The reduction in overall mammary gland size after 5 days of involution in $Plg^{-/-}$ mice was less than one-third the reduction seen in $Plg^{+/+}$ mice, and the reduction in secretory alveolar volume was almost one-thirtieth the reduction seen in $Plg^{+/+}$ mice. In fact, although $Plg^{-/-}$ mice had significantly less alveolar tissue than $Plg^{+/+}$ mice after 10 days of lactation, they ended up with significantly more alveolar tissue than their wild-type littermates after 5 days of involution.

Interestingly, heterozygous $Plg^{+/-}$ mice exhibited an intermediate phenotype in both lactation and involution, suggesting that a strict requirement for adequate levels of circulating Plg is not met by a single functional allele. Likewise, we have found that $Plg^{+/-}$ mice show a slight, but significant delay in incisional wound repair as compared to $Plg^{+/+}$ controls (our unpublished results), while $Plg^{-/-}$ mice show a more substantial delay (Rømer et al., 1996, Lund et al., 1999). Some cases of lactational failure in women, with its attendant potential for dire consequences in the breast-fed infant, have been attributed to insufficient glandular development of the breast (Neifert et al., 1985). Whether a subset of these cases is due to or exacerbated by low circulating Plg remains untested. Recently the inherited disease ligneous conjunctivitis has been linked with mutations in the *Plg* gene causing Plg deficiency (Schuster et al., 1997). Our results predict that some women with homozygous or heterozygous Plg deficiency would be unable to lactate, and that others would exhibit compromised lactation and involution.

How does plasminogen influence lactation and involution?

Because little is known concerning the role of ECM-degrading proteinases in establishing a normal lactational phenotype, it is difficult to speculate on why Plg is required. We can, however, rule out the possibility that Plg is required for the activation of other proteinases that are required for normal mammary gland development. Indeed, both gelatinase A and B were activated in lactating and involuting $Plg^{-/-}$ mice. Although activation of gelatinases A and B failed to occur in one study using cultured cells isolated from $Plg^{-/-}$ mice (Carmeliet et al., 1997), both enzymes were activated in another such study (Lijnen et al., 1998). Thus MMP-activating enzymes other than plasmin are likely to be present in vivo, but may not be accounted for in all cell culture experiments. Indeed, mast cell chymase has been shown to activate gelatinase B during skin tumor progression in vivo (Coussens et al., 1999). Thus, the proteolytic environment other than that provided by Plg appears intact in $Plg^{-/-}$ mice.

What then are the potential mechanisms leading to abnormal involution in $Plg^{-/-}$ mice? One possibility is that involution is not sensed. If this were the case, upregulation of the molecular program required for involution should be absent or diminished. We, however, observed that uPA and MMPs were upregulated normally. Thus, regulation of their expression during involution is independent of Plg concentration, and the

lack of excess expression suggests an absence of compensatory mechanisms. A second possibility is that although MMPs are upregulated, they are not activated in the absence of Plg; however, MMPs were activated. It is likely that Plg and MMPs play distinct roles in mammary development. One result of Plg deficiency is a change in proteolytic balance to one dominated by MMPs. Moreover, some aspects of the $Plg^{-/-}$ phenotype resemble those of excess MMP activity, such as fibrosis and adipocyte abnormalities (Thomasset et al., 1998; Sternlicht et al., 1999).

Another possible mechanism is a failure to sense apoptotic signals and/or the presence of excess survival signals. The most significant observation is that apoptosis was decreased in involuting $Plg^{-/-}$ mammary glands, indicating that this mechanism is involved. Although we cannot rule out systemic effects of Plg deficiency in involution, the mice appeared normal based on mass until 14 weeks, well after the experiments were terminated. In light of the potent fibrinolytic activity of Plg, it is also possible that the mammary epithelial cells do not sense the altered microenvironment because of increased accumulation of fibrin, which would substitute for basement membrane. Indeed, fibrin has been shown to support the growth of cultured mammary epithelial cells (Alford et al., 1998). Surprisingly, however, there was no excess fibrin in the $Plg^{-/-}$ mammary glands. This is probably the result of a significant, Plg-independent role for MMPs in fibrin degradation (Bini et al., 1996; Hiraoka et al., 1998; Lund et al., 1999) and the fact that fibrinolytic MMPs, such as Str-1 and MT1-MMP, are present during the late phase of mammary involution (Lund et al., 1996). In support of this premise, treatment of involuting $Plg^{-/-}$ mice with a broad-spectrum MMP inhibitor (GM6001) resulted in the accumulation of excess mammary fibrin (unpublished results). Thus, although rescue of the mammary phenotype in fibrin-deficient mice could not be tested directly, since fibrinogen-deficient female mice uniformly fail to carry their offspring to term (Suh et al., 1995), it appears unlikely that the accumulation of a provisional fibrin matrix is a significant contributor to the failure of $Plg^{-/-}$ mice to involute, whereas this is a major pathway for defective wound healing (Bugge et al., 1996; Rømer et al., 1996; Lund et al., 1999). Nevertheless, it is possible that subtle fibrin accumulation occurs in the $Plg^{-/-}$ mammary glands when and where the expression of other fibrinolytic enzymes is low. In this case, the fibrin may act as a nidus for granulation-like tissue and the formation of a fibrous interstitial matrix into which mammary epithelium is unable to grow and differentiate. This, in turn, may account for the tendency towards fibrosis in the $Plg^{-/-}$ mammary glands. Thus, unlike acute wound healing, fibrin accumulation may not be obvious in $Plg^{-/-}$ mammary glands at any particular instant, yet its repeated removal and replacement may help create an environment that is unfit for mammary development and lactation.

Another possible reason for insufficient involution is that the cellular environment is not changed appropriately in the absence of adequate Plg. Although basement membrane degradation takes place during involution, as indicated by cleavage of entactin/nidogen-1, there is an abnormal accumulation of breakdown products of this molecule, which could contribute to altered development and involution (Pujuguet et al., 2000). Thus, Plg either directly or indirectly contributes to the clearance of low molecular weight entactin cleavage products. Moreover, insufficient clearance of entactin breakdown products or of other bioactive ECM fragments may support cell viability and protect against cell death, and may thereby defy the process of mammary involution. Another intriguing observation was the altered stromal and adipocyte differentiation seen in Plg-deficient mice. This stromal phenotype may also be the result of abnormal accumulation of ECM fragments with cellular signaling capabilities. Such an environment may defy proper lactational development and may harbor signals that impede involution. Clearly, the epithelial and stromal compartments are coordinately regulated (Thomasset et al., 1998). In the present model, as long as the stromal differentiation remained closer to the lactational phenotype, epithelial involution was delayed.

Plasmin may also activate and release sequestered growth factors from the stromal ECM or cell surface. For example, plasmin can cleave high-affinity IGF-binding proteins (IGFBPs), cause the release of active IGF (Campbell and Andress, 1997) and bFGF (Brunner et al., 1991), and activate latent TGF-β (Munger et al., 1997). Thus, Plg may influence mammary function by regulating the availability or activation of important mammary growth and survival factors.

Our data demonstrate a functional role for plasmin(ogen) in mammary gland biology; particularly in postlactational involution. However, they also show that although involution is delayed by more than a week, it can ultimately proceed in the absence of Plg. There are strong similarities between the proteolytic mechanisms involved in mammary involution, wound healing and cancer invasion and metastasis (Johnsen et al., 1998), and the effect of Plg deficiency on these processes is also similar. Like mammary involution, skin wound healing and tumor metastasis are delayed but ultimately do occur in the absence of Plg (Rømer et al., 1996; Bugge et al., 1998; Lund et al., 1999). In the case of wound repair, there is functional overlap between Plg and one or more MMPs, such that a complete arrest of healing requires both Plg deficiency and MMP inhibition (Lund et al., 1999). Whether similar overlaps exist between Plg and other proteinases in mammary gland development and cancer invasion remain to be determined.

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