Matrilysin (Matrix Metalloproteinase-7) Selects for Apoptosis-resistant Mammary Cells in Vivo

Tracy Vargo-Gogola, Barbara Fingleton, Howard C. Crawford, and Lynn M. Matrisian

ABSTRACT

Overexpression of the matrix metalloproteinase matrilysin (matrix metalloproteinase-7) in the mouse mammary gland promotes mammary hyperplasia and accelerates the onset of oncogene-induced mammary tumors. In cell culture models, acute exposure of cells coexpressing Fas and Fas ligand (FasL) to matrilysin induces apoptosis, whereas chronic exposure to matrilysin selects for apoptosis-resistant cells. We now demonstrate that matrilysin promotes resistance to apoptosis in vivo. Matrilysin expression increased apoptosis in the involuting mammary gland of mice that had undergone a single pregnancy and lactation cycle. Premature basement membrane disruption was detected in matrilysin-expressing mice, which could account for the increase in apoptosis. However, multiparous mice, in which the involuting mammary epithelial cells have been repeatedly exposed to matrilysin, show a significant decrease in apoptosis. Mammary tissue from multiparous matrilysin-expressing mice showed decreased FasL expression, suggesting that loss of FasL is at least one mechanism of matrilysin-induced resistance to apoptosis. We propose that matrilysin promotes mammary tumor formation by enhancing the selection of cells that are resistant to apoptosis.

INTRODUCTION

The MMPs are a growing family of enzymes that have been implicated in nearly every stage of tumor progression, including growth, invasion, and metastasis (1). Matrilysin (MMP-7, E.C. 3.4.24.23) is expressed in many tumors of glandular epithelial origin including breast cancers (2–4). Unlike most MMPs, which are expressed by stromal cells, matrilysin is principally expressed by epithelial cells (5). We demonstrated previously that targeted expression of matrilysin to the mouse mammary gland resulted in hyperplasia in 50% (four of eight) of multiparous MMTV-MAT transgenic mice and significantly accelerated the onset of neu/ErbB2-induced mammary tumors (6). These results demonstrate that matrilysin plays a causative role in mammary tumor formation.

To elucidate the molecular mechanism(s) by which matrilysin expression contributes to tumor formation, we have focused on identifying substrates of matrilysin to which its tumor-promoting activity could be attributed. Previously, we reported two members of the tumor necrosis factor family, FasL and tumor necrosis factor-α, as substrates of matrilysin (7, 8). In the involuting rodent prostate, apoptosis of the prostate epithelium was shown to be partially dependent on the production of soluble FasL (7). In addition, we have demonstrated recently that acute treatment of mammary cells expressing FasL and its cognate receptor Fas with matrilysin induced apoptosis that was dependent on soluble FasL (9). Continual expression of matrilysin in the same cells selected for cells with reduced sensitivity to multiple apoptotic stimuli.

The purpose of our current study is to determine the effects of matrilysin expression on apoptosis of mammary epithelial cells in vivo. To do this, we investigated the effects of matrilysin expression on apoptosis in the postlactational involuting mouse mammary gland. In the mammary gland, apoptosis of the milk-producing epithelium occurs when the gland involutes after the cessation of lactation. Mammary gland involuion has been described as a two-stage process during which multiple apoptotic signaling pathways converge to remove the lactiferous epithelium, followed by remodeling of the gland to resemble its prepregnancy state (10). The first stage of mammary gland involution (days 1–4 after weaning) is protease independent, and the Fas apoptotic pathway has been shown to play a role in apoptosis during this stage (11). During the second stage of involution (days 4–10 after weaning), a number of proteases, including MMPs, are up-regulated and activated (10). It is at this time that degradation and remodeling of the BM underlying the epithelium occurs which serves to further promote removal of the milk-producing epithelium (12).

We hypothesize that matrilysin expression during multiple apoptotic cycles in successive rounds of mammary gland involution selects for cells with reduced sensitivity to apoptosis. Our previous observations demonstrating that matrilysin alters Fas-dependent apoptosis in vitro suggest that matrilysin expression could affect apoptosis in vivo by modulating signaling through the Fas pathway. We propose that inhibition of apoptosis by matrilysin is one mechanism by which matrilysin promotes the formation of the hyperplastic lesions that were detected in multiparous MMTV-MAT mice but not in nontransgenic mice of the same parity.

MATERIALS AND METHODS

Animal Models. MMTV-MAT (13) or wild-type (FVB) mice, 8–10 weeks of age, were allowed to go through one or three pregnancies and lactation cycles to generate singly parous or multiparous mice, respectively. Mice lactated for 9 days to establish full lactation, and then the pups were removed to induce involution. FVB mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Histology, Ultrastructural, TUNEL, and Active Caspase-3 Analysis. For histological analysis, glands were excised, fixed in 4% paraformaldehyde, dehydrated through ethanol, and embedded in paraffin. Five-μm sections were stained with H&E. Apoptosis indices were determined using the Apoptag kit (Intergen, Purchase, NY) according to the manufacturer’s instructions. For in situ analysis of caspase-3 activity, tissue sections were stained according to the manufacturer’s protocol with a rabbit anticleaved caspase-3 antibody (Asp175) diluted 1:64 (Cell Signaling Technology, Beverly, MA). An average of 4000 nuclei from four to six mice/group were scored. Student’s t test was used to determine statistical significance. For TEM, mammary tissue from two wild-type and two MMTV-MAT multiparous mice was processed and analyzed as described previously (14).

Western Blotting. Mammary gland lysates were prepared as described previously (6). Protein (325 μg) was electrophoresed through a SDS-15% polyacrylamide gel and transferred to polyvinylidene difluoride membrane for Western blotting. Western blotting was performed with rat anti-FasL (1:500; HC-11; Alexis Biochemicals, San Diego, CA), rabbit anti-Fas (1:200; X-20; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-Bax or anti-Bcl-x (1:1000; P19 and S18; Santa Cruz Biotechnology). For Western analysis,
membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 5% milk. Membranes were then incubated for 2 h with primary antibodies diluted in 5% milk in TBS + 0.05% Tween (TBST). Membranes were washed in TBST and then incubated for 1 h with biotinylated secondary antibodies, followed by a 30-min incubation with a peroxidase-conjugated streptavidin antibody. Blots were developed using Western Lightning Chemiluminescent Reagent according to the manufacturer’s instructions (Perkin-Elmer, Boston, MA).

**RNA Preparation and RT-PCR.** RNA was prepared from mammary tissues using the TRIZol method (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RT-PCR of Matrilysin and glyceraldehyde-3-phosphate dehydrogenase was performed as described previously (8). Reactions performed with cDNA generated in the absence of reverse transcriptase were negative.

**RESULTS**

To investigate the effects of matrilysin expression on apoptosis we used transgenic mice that express matrilysin in the mammary epithelium under the control of the MMTV long terminal repeat (MMTV-MAT mice; Ref. 13). Previous analysis demonstrated that targeted expression of matrilysin to the mouse mammary gland resulted in precocious mammary gland development in virgin mice and mammary hyperplasia in multiparous mice. In this study, we first determined whether the matrilysin transgene was expressed during postlactational mammary gland involution. Wild-type and MMTV-MAT transgenic mice were allowed to go through pregnancy and establish lactational mammary gland involution. Mammary glands were collected at 1, 3, and 5 days after forced weaning, and RNA was prepared from the tissue. RT-PCR revealed that the matrilysin message was present at all time points analyzed in the MMTV-MAT mice but not in the FVB wild-type mice (Fig. 1, A and B).

To assess the effects of matrilysin expression on postlactational mammary gland involution after a single pregnancy and lactation cycle, histological analysis was performed on mammary glands collected at days 1, 3, and 5 after forced weaning. This analysis revealed that matrilysin expression had no apparent effect on the gross characteristics of mammary gland involution (Fig. 1C). Collapse of the lactiferous alveoli and repopulation of the glands with adipocytes proceeded at a similar rate in both the MMTV-MAT transgenic and wild-type mice.

As previous data had indicated a role for matrilysin in apoptosis, TUNEL analysis was performed on mammary tissue collected at days 1, 3, and 5 of involution to determine whether there were any effects in this system. Interestingly, MMTV-MAT mice showed a significant increase in the apoptotic index (10.2 ± 0.74% versus 5.1 ± 0.91%; \( P = 0.0006 \)) 3 days after forced weaning compared with wild-type mice. No significant difference in apoptosis was detected at the other time points analyzed in the MMTV-MAT mice but not in the FVB wild-type mice (Fig. 1, A and B).

To explore the mechanism of matrilysin-induced apoptosis, we examined the involuting mammary gland by TEM. Previously, overexpression of a related MMP, stromelysin-1, was shown to induce apoptosis of the mammary epithelium in the developing mammary gland (14, 15). Normally during the first stage (days 1–4) of mammary gland involution, BM integrity is maintained (10). At day 4 of involution, MMP expression and activation increases, and BM degradation and remodeling of the extracellular matrix begins. Degradation of the BM serves to promote further regression of the mammary epithelium (16). To determine whether matrilysin expression resulted in premature disruption of the BM in the first stage of mammary involution, mammary tissues from MMTV-MAT transgenic and wild-type mice at day 3 of involution were analyzed by TEM. Compared with wild-type mice, the BM in MMTV-MAT mice was disorganized, and the lamina densa was absent in focal areas throughout the gland (Fig. 4). These data demonstrate that aberrant expression of matrilysin during mammary gland involution results in premature disruption of the BM that correlates with an increase in apoptosis.

To test our hypothesis that persistent expression of matrilysin during multiple postlactational apoptotic cycles selects for cells with
reduced sensitivity to apoptosis, we generated multiparous MMTV-MAT transgenic and wild-type mice that had gone through three pregnancy and lactation cycles. At day 9 of the third lactation cycle, the pups were removed to trigger mammary gland involution. Mice were euthanized at 3 and 5 days after removal of the pups, and mammary glands were excised. Interestingly, TUNEL analysis revealed a significant decrease in the apoptotic index of matrilysin-expressing mice compared with control mice at day 3 of involution (8.6/11006 1.0% versus 12.6%/11006 1.6; P/11005 0.046; Fig. 2C). Similar to the results from the mice that underwent a single pregnancy and lactation cycle, the effects on apoptosis were limited to day 3 of involution. The results of the TUNEL analysis were confirmed by in situ analysis for active caspase-3 (Fig. 3). By this method, MMTV-MAT mice also showed reduced apoptosis, indicated by a decrease in the number of active caspase-3-positive cells compared with wild-type mice (4.83/11006 0.75% versus 10.4/11006 1.69%; P = 0.021).

After a single pregnancy, MMTV-MAT mice showed a 2-fold increase in apoptosis by TUNEL analysis compared with wild-type animals 3 days after removal of pups (Fig. 2D). In contrast, after three pregnancies, this relationship was reversed, and MMTV-MAT mice showed a 0.68-fold decrease in apoptosis compared with wild-type mice. By combining these data, MMTV-MAT mice showed a 2.93-fold (2/0.68) difference relative to the wild-type mice in the response of the mammary epithelium to involution-induced apoptosis after multiple pregnancies. These results support the conclusion that repeated exposure of apoptotic mammary epithelial cells to matrilysin in vivo selects for cells with reduced sensitivity to apoptosis.

To explore the mechanism underlying the reduction in apoptosis seen in the multiparous MMTV-MAT mice, the expression of Bcl-family members that have been shown to be involved in mammary gland involution was analyzed (17). Western blotting for Bcl-x and Bax was performed on mammary tissue lysates from three individual MMTV-MAT and wild-type mice. No significant difference in expression of Bax or either of the forms of Bcl-x was detected (Fig. 5).

The Fas apoptotic pathway has been demonstrated to play a role during the first stage of mammary gland involution. Mice expressing a nonfunctional form of FasL (gld mice) or a mutant Fas receptor (lpr mice) showed a significant delay in the onset of apoptosis after forced weaning (11). In both in vitro and in vivo models, we have observed that matrilysin affects FasL-dependent apoptosis (7, 9). We, therefore,
hypothesized that repeated rounds of matrilysin expression driven by multiple involution cycles could result in altered signaling through the Fas apoptotic pathway. To test this, we wanted to determine whether FasL expression is altered during involution in multiparous MMTV-MAT transgenic mice. Western analysis was performed on lysates prepared from mammary tissue from three different wild-type or MMTV-MAT mice collected 3 days after forced weaning. Expression of the receptor, Fas, was detected in all mice (data not shown). Interestingly, mammary tissue from two of the three multiparous MMTV-MAT mice showed a reduction in expression of full-length FasL in comparison with wild-type mice (Fig. 5). These data suggest that the reduction in apoptosis observed in the multiparous MMTV-MAT mice is, in part, attributable to decreased FasL expression.

DISCUSSION

Matrilysin expression in the mammary gland has been reported previously to result in the development of mammary hyperplasias and accelerate the onset of neu/erbB2-induced mammary tumors (6). Recently, we have attempted to elucidate the cellular and molecular mechanisms by which matrilysin promotes tumor formation. To this end, we have demonstrated that acute exposure of early-stage mammary tumor cell lines to matrilysin induced apoptosis, whereas continual expression of matrilysin in the same cells selected for cells that were less sensitive to multiple apoptotic stimuli (7). These results prompted us to investigate whether matrilysin could also select for cells that are less sensitive to apoptosis in vivo. Here we show that expression of matrilysin in the mouse mammary gland over multiple pregnancy and involution cycles selects for cells that are less sensitive to apoptosis. After a single pregnancy, which allowed for an acute exposure of the mammary epithelial cells to matrilysin, MMTV-MAT mice showed increased apoptosis during involution. However, after multiple pregnancies or repeated exposures of the involuting mammary epithelium to matrilysin, MMTV-MAT mice showed a significant reduction in apoptosis compared with wild-type mice.

On the basis of cell culture models, we have suggested that the apoptosis-enhancing effect of matrilysin results in the elimination of normal cells with a fully functional apoptotic response and the retention of a subpopulation of cells with an aberrant or attenuated response to death-inducing signals. Repeated or chronic expression of matrilysin in an apoptotic environment thus facilitates the clonal evolution of a population of cells that can circumvent the normal programmed cell death pathways. These cells are then able to accumulate additional oncogenic mutations, resulting in tumor progression. Our results in the involuting mammary glands of matrilysin-
expressing mice demonstrate that mammary epithelial cells respond to the apoptotic effects of matrilysin similarly both in vitro and in vivo. These data suggest that the induction of mammary hyperplasias and acceleration of oncogene-induced tumorigenesis in the MMTV-MAT mice are the result of the ability of matrilysin to select for apoptosis resistance in vivo.

Previous studies have shown that matrilysin affects epithelial cell apoptosis through cleavage and solubilization of FasL (9, 18). The Fas-apoptotic pathway has been shown recently to be involved in mediating apoptosis during the first stage of mammary gland involution (11). Therefore, we examined the expression of FasL in mice that have undergone multiple pregnancies in an attempt to identify the molecular mechanism underlying the resistance to apoptosis. In contrast to multiparous wild-type mice, two of three multiparous MMTV-MAT transgenic mice showed decreased FasL expression. Thus, selection of cells with reduced expression of FasL in multiparous MMTV-MAT transgenic mice is a potential mechanism by which chronic matrilysin exposure could result in decreased apoptosis. However, at least one multiparous MMTV-MAT mouse expressed FasL at levels comparable with wild-type animals, and our previous in vitro results demonstrated that mammary cells showed no alterations in FasL levels in response to chronic matrilysin expression (9). Therefore, it is likely that there are multiple mechanisms of matrilysin-induced resistance to apoptosis. We observed no consistent differences in the expression levels of the Bcl family members examined. A number of other genes are reportedly involved in mammary gland involution, including the transcription factors p53, STAT3 and STAT5, and nuclear factor-κB (17). Alterations in any one of these factors could potentially contribute to the resistant phenotype, and individual multiparous MMTV-MAT mammary glands could be composed of subpopulations of cells with different mechanisms of resistance.

In MMTV-MAT transgenic mice, matrilysin protein is detected throughout the mammary epithelial cells, suggesting that matrilysin may come into contact with BM substrates as well as cell surface substrates (13). Indeed, our TEM results indicate that the BM of MMTV-MAT mice is degraded prematurely, suggesting that matrilysin may also target mammary epithelial cell survival by disrupting cell-matrix contacts. BM disruption during the first stage of mammary gland involution could result in increased cell death by inducing anoikis, which is triggered by loss of cell-matrix contacts. Alternatively, BM degradation could release proteins that modulate apoptosis. Matrilysin may therefore, enhance apoptosis in the mammary gland through cleavage of both cell surface (e.g., FasL) and BM substrates. We suggest that both mechanisms are likely to be relevant to MMP-enhanced mammary tumorigenesis, because the selection of apoptosis-resistant cells would occur irrespective of the mechanism by which apoptosis is induced. This conclusion is supported by the observation that mice expressing stromelysin-1 (MMP-3) in the mammary gland also showed aberrant disruption of the BM, which had a significant effect on survival of the mammary epithelium (14, 15). These mice demonstrated a dramatic incidence of mammary tumors that is attributed to the matrix-degrading properties of stromelysin-1 (19, 20).

The data reported here demonstrate for the first time that repeated exposure of apoptotic cells in vivo to a MMP can select for cells that are less sensitive to death-inducing stimuli. These results imply that inhibition of matrilysin expression may be effective in retarding tumor progression if it is continuously administered beginning at early stages of carcinogenesis. The synthetic MMP inhibitors have, in general, been ineffective in the treatment of advanced cancer (21). There is evidence that the treatment of early-stage tumors with an MMP inhibitor is more efficacious than treatment of late-stage tumors (22). The ability of broad-spectrum MMP inhibitors and genetic elimination of matrilysin to reduce the number of intestinal adenomas also supports the use of MMP inhibitors in early-stage tumors (23, 24). On the basis of these observations, MMP inhibition may be an effective chemopreventive strategy.

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