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The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis

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Background: The Fas ligand/Fas receptor (FasL/Fas) system is an important mediator of apoptosis in the immune system where the juxtaposition of cells expressing the cell-surface ligand induces the apoptotic pathway in Fas-expressing lymphocytes. The FasL/Fas system has also been shown to be involved in apoptosis in epithelial tissues, including the involuting rodent prostate. FasL can be shed through the action of an hitherto unidentified metalloproteinase to yield soluble FasL (sFasL), although the biological activity of sFasL has been disputed.

Results: Here we report that the matrix metalloproteinase matrilysin can process recombinant and cell-associated FasL to sFasL, and that matrilysin-generated sFasL was effective at inducing apoptosis in a target epithelial cell population. In the involuting mouse prostate, FasL and matrilysin colocalized to the cell surface in a restricted population of epithelial cells. Mice deficient in matrilysin demonstrated a 67% reduction in the apoptotic index in the involuting prostate compared with wild-type animals, implicating matrilysin in this FasL-mediated process.

Conclusions: These results show that a functional form of sFasL was generated by the action of the metalloproteinase matrilysin, and suggest that matrilysin cleavage of FasL is an important mediator of epithelial cell apoptosis.

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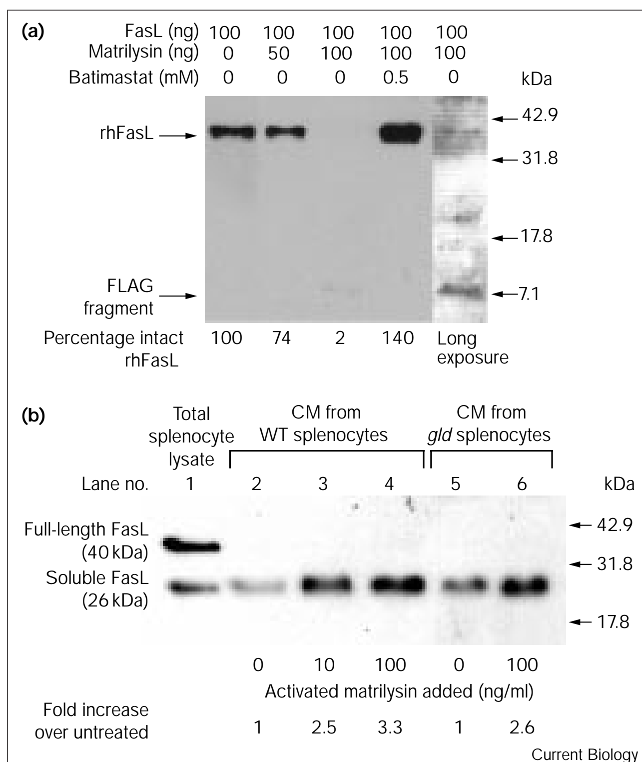
Background

The Fas ligand/Fas receptor (FasL/Fas) system was initially identified as an important mediator of apoptosis in the immune system, in which interaction with cells expressing cell-surface FasL induces the apoptotic pathway in Fas-expressing lymphocytes [1]. A number of studies have identified non-lymphoid tissues expressing both Fas and FasL [2,3], however, and it has been suggested that precise spatial and temporal regulation of the FasL–Fas interaction controls remodeling in these tissues. Recently, the FasL/Fas system has been implicated in prostate involution. The withdrawal of androgen by physical or chemical castration initiates the involution of the rodent prostate. There is an overall reduction in organ size, apoptosis of the prostate epithelial cells, and a change in the composition of the gland to contain primarily connective tissue components [4]. Fas levels have been reported to increase in the mouse prostate following castration, and in *lpr* mice deficient in functional Fas, androgen withdrawal is followed by inhibition of involution and failure to induce DNA fragmentation [5]. In the involuting rat ventral prostate, both Fas and FasL were reported to be induced following castration and to decline after the process was completed [6,7]. The requirement for FasL–Fas interactions in involuting prostate epithelial cells presents a topological problem regarding the accessibility of a cell-surface ligand to a receptor when both are positioned on the apical surface of cells that are anchored to a basement membrane. A potential solution to this conundrum is that FasL can be processed to a soluble form (sFasL) by an as-yet-unidentified metalloproteinase [8,9]. Both the transmembrane and soluble forms of human FasL have been shown to be active in inducing apoptosis in lymphoid cell targets. Under the same conditions, however, murine sFasL has been reported to be nonfunctional [10–12]. Thus, the mechanism of Fas stimulation in the involuting rodent prostate is unclear.

We have previously reported that the matrix-degrading metalloproteinase matrilysin (MMP-7, PUMP-1, EC 3.4.24.23) is strongly induced in the epithelium of the rat ventral prostate during involution [13]. Matrilysin is distinct from the other known MMP family members in that it is preferentially expressed by cells of glandular epithelium rather than in connective tissues [14]. In addition to its activity on basement membrane and extracellular matrix components, matrilysin has been reported to cleave a variety of secreted and cell-surface proteins [14]. These cell-surface substrates include the transmembrane precursor to tumor necrosis factor-α (TNF-α), a member of the same protein family as FasL. Cleavage of the TNF-α precursor by matrilysin and several other metalloproteinases

Figure 1



Generation of soluble FasL (sFasL) by matrilysin. **(a)** Recombinant human FasL (rhFasL,100 ng) was incubated with varying amounts of APMA-activated matrilysin with or without 0.5 mM Batimastat for 30 min at 37°C. The reactions were analyzed by western blotting for the FLAG tag on the rhFasL. The FLAG-tagged fragment was apparent upon a longer exposure. The amount of intact rhFasL in matrilysin-treated samples relative to the untreated sample was determined by densitometry and the percentage remaining is indicated at the bottom of each lane. The blot shown is representative of two separate experiments. **(b)** Activated splenocytes (either wild type (WT) or *gld*) were treated for 6 h with increasing concentrations of activated recombinant matrilysin. The conditioned media (CM) were collected and filtered before analysis of sFasL by western blotting. The fold increase of sFasL detected in treated versus untreated splenocytes is indicated along the bottom. A sample of a total splenocyte lysate is also included to indicate the relative sizes of the full-length and soluble forms of FasL. Sizes of molecular weight markers are indicated on the right-hand side. The blot shown is representative of six experiments.

can result in the release of active ligand from the cell surface [15]. Although the processing of both TNF-α and FasL is efficiently blocked by synthetic MMP inhibitors, it is not affected by the endogenous regulators of MMP activity, the tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2 [16,17]. This suggested that the relevant 'sheddase' is a metalloproteinase of a different subfamily. In fact, the TNF-α converting enzyme, TACE, was identified as a member of the ADAM (a disintegrin and metalloproteinase) family of metalloproteinases, a group distinct from the MMPs [18,19]. Within the MMP family, however, matrilysin is the only known member that lacks the hemopexin domain thought to be important in interactions with TIMPs and is thus believed to be less sensitive to the inhibitory action of these proteins [20]. These observations, combined with the requirement for Fas in prostate involution, led us to design experiments to determine if FasL is a matrilysin substrate and if matrilysin is functionally involved in prostate involution. Our results indicate that matrilysin can proteolytically process murine FasL to an active soluble form and that tissue involution requiring FasL-mediated apoptosis is attenuated by the lack of this enzyme.

Results

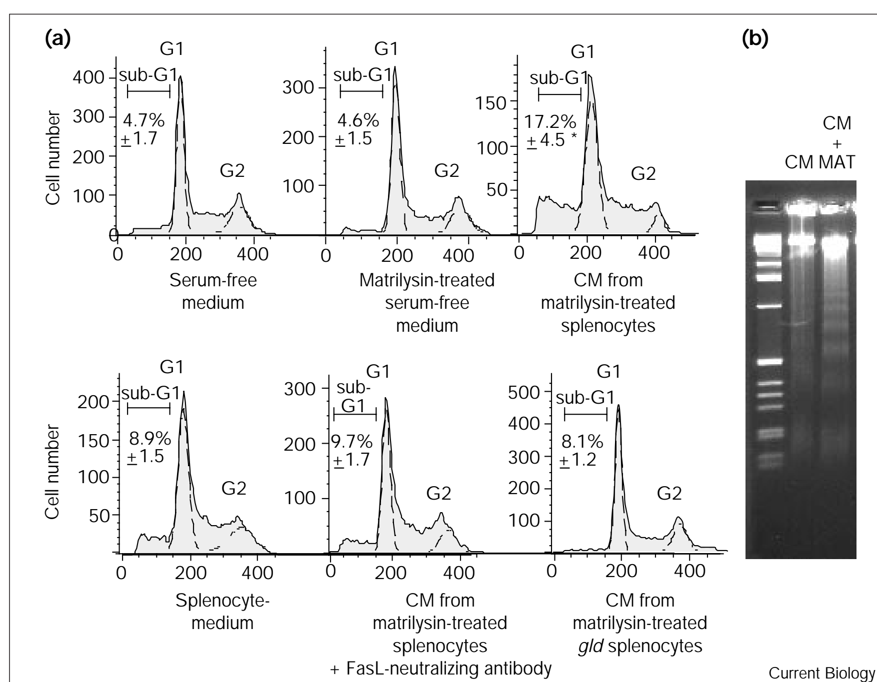
FasL is a direct substrate of matrilysin

To determine if FasL is a matrilysin substrate, purified recombinant human matrilysin was activated and incubated with FLAG-tagged recombinant human FasL (rhFasL). Intact and cleaved FasL was detected by western blot analysis using an anti-FLAG antibody. Matrilysin, at an approximately 1:1 molar ratio to rhFasL, efficiently cleaved > 90% of rhFasL in 30 minutes (Figure 1a). Cleavage was completely inhibited in the presence of the synthetic MMP inhibitor Batimastat (Figure 1a). To determine if a similar cleavage event occurred with FasL in its native conformation on the cell surface, murine splenocytes were treated with concanavalin A and interleukin-2 for 8 hours to induce lymphocyte FasL expression [10]. The FasL-expressing splenocytes were incubated with activated recombinant matrilysin for 6 hours and the conditioned medium was analyzed for FasL by western blotting. Matrilysin treatment increased the amount of the 26 kDa soluble form of FasL in conditioned medium in a dose-dependent manner that was inhibited by the addition of 5 μM Batimastat (Figure 1b and data not shown). Splenocytes isolated from mice homozygous for the *gld* mutation, an inactivating point mutation in the FasL gene that abrogates FasL interaction with the Fas receptor [21], were cleaved similarly, indicating that the mutation in FasL in these cells does not affect its ability to be proteolytically processed.

sFasL induces apoptosis in epithelial cells

The sFasL released by matrilysin cleavage was assayed for apoptosis-inducing ability using human embryonic kidney 293 cells as indicators. The 293 cells represent a non-tumor epithelial cell line that expresses Fas and is sensitive to Fas-mediated apoptosis [22]. The 293 cells were incubated for 22 hours with conditioned medium from splenocytes treated or not treated with activated matrilysin and apoptosis was measured by determining the percentage of cells with fragmented nuclei containing a sub-G1 content of DNA. Matrilysin-treated splenocytes gave conditioned medium that demonstrated a reproducible 3.7-fold increase in apoptosis compared with control medium (Figure 2a, $p < 0.05$), corresponding to the increase in the levels of sFasL (Figure 1b, lane 4). The effect on 293 cells of conditioned medium from matrilysin-treated splenic

Figure 2



sFasL is functionally active. **(a)** Splenocyte conditioned medium (CM) in the presence of a FasL-neutralizing antibody or an isotype control was incubated with 293 cells for 22 h. The epithelial cells were harvested, fixed and stained with propidium iodide and then analyzed by flow cytometry. Peaks corresponding to populations in G1 and G2 phases of the cell cycle are indicated, as are the sub-G1 areas. The numbers on each panel correspond to the mean ± standard deviation (SD) of the population with fragmented DNA (sub-G1) obtained from at least three separate experiments. Statistical significance was assessed using Student's *t*-test and those samples reaching significance ($p < 0.05$) are indicated with an asterisk. **(b)** Genomic DNA was isolated from 293 cells which had been exposed to untreated splenocyte CM (CM) or CM from splenocytes treated with 100 ng/ml matrilysin (CM + MAT) and was analyzed by agarose gel electrophoresis. The characteristic DNA laddering pattern associated with apoptosis can be clearly seen in the matrilysin-treated CM lane. The left-hand lane contains a DNA size marker. The gel shown is representative of three separate experiments.

blasts was confirmed to be apoptosis using a DNA laddering assay (Figure 2b). Direct addition of matrilysin to 293 cells had no effect on apoptosis (Figure 2a). Stimulation of apoptosis was also observed using HBL100 human breast epithelial indicator cells (data not shown).

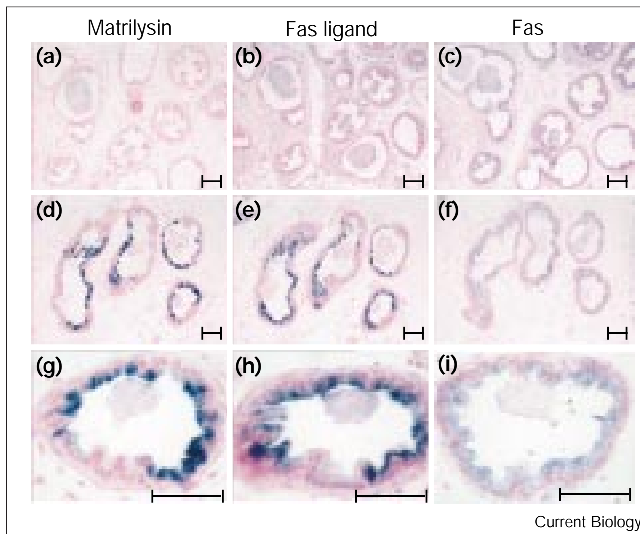
To ensure that the matrilysin-induced increase in apoptosis was the result of FasL cleavage, spleen cells from FasL-defective *gld* mice were used as a source of sFasL. Conditioned medium from matrilysin-treated *gld* cells did not induce apoptosis (Figure 2a), although comparable amounts of sFasL were released from these cells and from wild-type cells following matrilysin treatment (Figure 1b, compare lanes 4 and 6). In addition, the effect of matrilysin was abrogated when conditioned medium from matrilysin-treated wild-type splenocytes was incubated with the FasL-neutralizing antibody Kay-10 (Figure 2a). Thus, the effect of matrilysin treatment on activated splenocytes depended on the generation of active soluble FasL. Conditioned medium from splenocytes that had not been treated with matrilysin induced only a 1.9-fold increase in apoptosis (Figure 2a), which was not significantly different from the basal apoptotic index in 293 cells. This small increase might reflect the constitutively released sFasL present in the conditioned medium (Figure 1b, lane 2) but the more probable explanation for this induction, given that the effect is similar whether *gld* spleen cells or the FasL-neutralizing antibody is used, is the presence in the conditioned media of other death factors.

Matrilysin and FasL are coexpressed in the prostate

We have shown previously that matrilysin mRNA is induced in epithelial cells in the rat ventral prostate following castration [13]. To determine whether matrilysin protein is similarly regulated in the mouse dorsal/lateral (DL) prostate, adult male mice were castrated and prostrates were removed at days 2 and 5 post-castration and examined by immunohistochemistry with an affinity-purified anti-mouse matrilysin polyclonal antibody. There was little or no matrilysin protein observed in the DL prostrates of intact animals (Figure 3a). By 2 days post-castration, significant matrilysin production was observed in individual glands and expression continued for 5 days after castration (Figure 3d and data not shown). Similarly to *in situ* hybridization observations in the rat [13], murine matrilysin protein was restricted to a subpopulation of epithelial cells of the prostate (Figure 3d,g). Interestingly, the matrilysin protein localized to the apical surfaces of the prostate epithelial cells (Figure 3g), rather than concentrating at the basement membrane as would be expected for a matrix-degrading protease. Apical and luminal localization of matrilysin has previously been observed in various human glandular epithelia [23].

FasL and Fas levels were also examined by immunohistochemistry in the involuting mouse prostate. There was little or no FasL detected in the normal mouse prostate (Figure 3b), consistent with low levels of FasL in the mouse prostate reported by French *et al.* [2]. As observed with

Figure 3



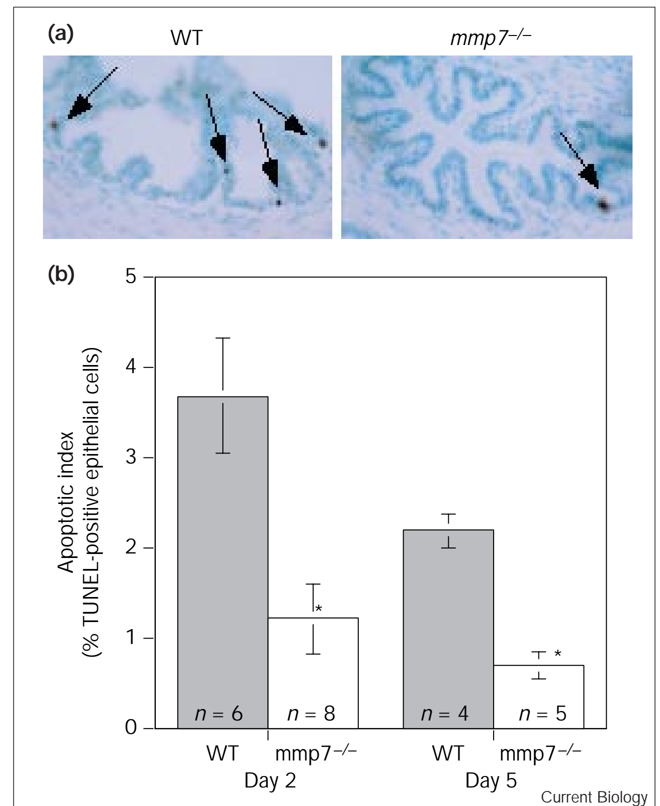
Immunohistochemical localization of matrilysin, FasL and Fas in control and castrated mice. Immunohistochemical (IHC) staining was performed on dorsal/lateral prostate tissue from control **(a–c)** and 2-day castrated **(d–i)** wild-type mice. IHC staining for matrilysin (a,d,g), FasL (b,e,h) and Fas (c,f,i) is shown using a blue substrate to detect HRP and a red nuclear counterstain. The scale bar represents 50 μm.

matrilysin, however, there was a strong induction of FasL in the epithelium of the prostate 2 days after castration, which continued through 5 days post-castration (Figure 3e,h and data not shown). Interestingly, matrilysin and FasL demonstrated a virtually identical pattern of spatial colocalization in adjacent sections (Figure 3, compare d and e, g and h). In contrast to matrilysin and FasL, Fas was present in normal mouse prostate epithelium at a low constitutive level that remained unchanged following castration (Figure 3c,f and data not shown). Fas expression was more widespread that that of matrilysin or FasL, and all prostate epithelial cells had detectable Fas immunoreactivity, which also appeared localized to the apical surface (Figure 3i). Although Fas has been previously reported to be upregulated in the murine prostate in response to castration [5], we could detect no difference in Fas levels by immunohistochemistry following castration (Figure 3c,f,i).

Matrilysin-null mice demonstrate reduced apoptosis

To determine if matrilysin regulates Fas-mediated prostate apoptosis, castration-induced apoptosis was compared in wild-type and matrilysin-deficient mice. Matrilysin-deficient (*mmp7*$^{-/-}$) mice previously generated in this laboratory develop normally and have no obvious reproductive defects, although alterations in intestinal tumor development and compensatory increases in other MMP family members during uterine involution have been observed [24,25]. The apoptotic index in the DL prostate of wild-type and *mmp7*$^{-/-}$ mice was examined at 2 and 5 days post-castration using the TUNEL assay, which detects fragmented nuclear DNA (Figure 4a). The lack of matrilysin in the *mmp7*$^{-/-}$ mice was confirmed by immunohistochemical analysis of the involuting prostate and the expression of Fas and FasL was identical to that observed in wild-type mice (data not shown). At both 2 and 5 days post-castration, there was a highly significant 67% reduction ($p < 0.001$) in the percentage of TUNEL-positive epithelial cells in the *mmp7*$^{-/-}$ mice compared with wild-type animals (Figure 4b). As judged by the histological morphology of the DL prostate in matrilysin-deficient mice after castration, involution occurred but appeared to be slightly slower than in the matrilysin wild-type animals (data not shown). Two days post-castration the *mmp7*$^{-/-}$ prostrates were larger than wild type as determined by wet weight analysis, but the difference did not reach statistical significance. The reduction in apoptosis caused by the loss of matrilysin

Figure 4



Castration-induced apoptosis in wild-type and matrilysin-deficient animals. **(a)** The TUNEL assay was performed on dorsal/lateral prostate tissue at 2 and 5 days post-castration in wild-type (WT) and matrilysin-deficient animals (*mmp7*$^{-/-}$). An example of the staining seen at 2 days post-castration in WT and *mmp7*$^{-/-}$ glands is shown. Arrows indicate TUNEL-positive cells. **(b)** Epithelial nuclei (> 3,000 for each mouse) were counted and the percentage of TUNEL-positive epithelial cells was determined. There was a statistically significant 67% reduction in TUNEL-positive cells in the matrilysin-deficient animals at both 2 and 5 days after castration (two-tailed *t*-test, $^*p < 0.001$). n = number of animals in each group. Error bars are ± SD.

indicates that matrilysin activity contributes to the removal of epithelial cells during prostate regression.

Discussion

The MMPs or their endogenous inhibitors have been implicated in apoptotic processes in several systems [26–31]. In general, this activity is thought to occur as a result of the breakdown of cell–matrix contacts leading to a form of apoptosis referred to as anoikis [32,33]. In the involuting prostate matrilysin is localized to the apical surface of the cells, however, suggesting that substrates that are also apically localized are more relevant. Apoptosis of the prostate epithelium has been shown to be dependent on a Fas-mediated pathway. Here we provide evidence that, in contrast to a role in destroying matrix-mediated survival signals, the MMP matrilysin can promote apoptosis via the generation of a biologically active death-inducing protein, sFasL.

The activity of sFasL has been extensively debated in the literature. There is evidence both in favor of and opposing its activity. We hypothesize that activity is target-cell specific. In general, sFasL with little or no activity has been reported when lymphoid cells have been used as targets, for example the human T-cell line Jurkat [34] or murine T-cell lymphoma WR19L and its Fas-expressing transformant W4 [10,11]. This might be because of an activation-induced resistance to sFasL as described previously [11]. In contrast, epithelial cell populations, for example hepatic stellate cells, have been reported to be sensitive to sFasL-mediated death [35]. We demonstrated that matrilysin-generated sFasL was effective at inducing apoptosis in target epithelial cell populations. The same preparation had no activity when Jurkat T cells were used as the indicator cells (data not shown). This observation supports the premise that the apoptosis-inducing activity of sFasL is dependent on the target cell population.

The MMPs have been implicated as sheddases for biologically active cell-surface factors or receptors [36,37]. We demonstrated that matrilysin can cleave recombinant as well as cellular FasL, supporting the possibility of a direct proteolytic effect. In a recent phage-display study a number of excellent peptide substrates for matrilysin were found, all of which contained the core sequence Glu–Leu–Arg [38]. Interestingly, this sequence is found in mouse, rat and human FasL and is highly conserved at a position close to the transmembrane domain, suggesting that it might represent the matrilysin-cleavage site. Although direct cleavage appears probable, we cannot rule out the possibility that a cascade of enzymes is required for the generation of sFasL.

Our data indicate that the loss of matrilysin reduces prostate epithelial cell apoptosis by 67%, but does not completely abolish the effect of androgen ablation in the prostate. There are several possible explanations for the continued low-level apoptosis in the matrilysin-deficient mice and the final completion of the involution process, including the presence of other proteases that might also cleave FasL to its soluble form. We have preliminary evidence that the MMPs gelatinase A, gelatinase B and stromelysin-1 are not capable of releasing sFasL (data not shown). Other metalloproteinases, particularly those similar to the TNF-α sheddase TACE, are possible candidates, however. We also cannot rule out the possibility that the induction of precursor FasL is sufficient to initiate some interactions with Fas at the cell surface, bypassing the need for FasL cleavage. Finally, redundant pathways for apoptosis induced by androgen withdrawal are also possible. For example, incomplete elimination of apoptotic bodies in the involuting prostrates of p53-deficient mice demonstrates that both p53-dependent and independent mechanisms of apoptosis exist in this system [39].

Fas-mediated apoptosis is well studied as a mechanism for selective removal of T cells following an immune response. Recently, the expression of Fas and FasL was examined in a number of non-lymphoid tissues [2,3]. Fas was expressed in almost all tissues examined, whereas FasL expression was much more restricted. Interestingly, both of these studies identified a number of normal tissues that express both Fas and FasL, including lung, intestine, seminal vesicle and prostate. This colocalization suggests that either Fas and FasL are expressed by different cell types within the same tissue, or that coexpression of FasL and Fas on the same cell does not initiate apoptosis in the absence of other signals. One possibility is that effective FasL–Fas interactions on the same cell type are dependent on the production of soluble FasL. This might be particularly important for epithelial cells, which are attached to a basement membrane and sort membrane components into distinct compartments. In the glandular epithelium of the prostate, the transmembrane proteins Fas and FasL are both apically localized. Although lateral interactions are possible, complete interaction between their extracellular domains would presumably be constrained by transmembrane anchoring. Our results suggest that the production of an epithelium-specific processing enzyme such as matrilysin might be necessary to initiate an apoptotic signaling pathway in solid tissues.

Materials and methods

Animals

The generation of matrilysin-null mice was described previously [24]. Outbred (Sv129 × C57/Bl6) mice homozygous for the $mmp7^{m1Vu}$ or wild-type *mmp7* alleles were used for all castration studies. Ten-week-old male mice were anesthetized and castrated via the abdominal route. The animals were killed 2 and 5 days after castration and the dorsal/lateral prostate was removed, fixed in 4% paraformaldehyde overnight and paraffin embedded.

Cleavage of FasL

Purified recombinant human soluble FasL (entire extracellular region attached to a FLAG tag) was obtained from Alexis Biochemicals (San

Diego, CA) and the purified human matrilysin was a generous gift from Harold VanWart (Roche Biosciences Inc., Palo Alto, CA) [40]. The matrilysin protein was activated by treatment with 1 μM pAminophenyl mercuric acetate (APMA) for 1 h at 37°C. The indicated amounts of FasL and activated matrilysin were added to 30 μl MMP buffer (50 mM Tris pH 7.4; 10 mM $CaCl_2$) and incubated at 37°C for 30 min. The MMP inhibitor Batimastat was a generous gift from Peter Brown (British Biotech, Oxford, UK) and was added at a concentration of 0.5 mM.

Freshly isolated spleens from C57/Bl6 mice were macerated, the cells suspended in RPMI 1640 medium (Gibco-BRL) and centrifuged to remove debris. Splenocytes were isolated following removal of red blood cells by hydrolysis and rinsing in PBS and RPMI 1640 and were resuspended in RPMI 1640 supplemented with 10% FCS (Atlanta Biologicals). Activation was achieved by incubation with 100 μg/ml concanavalin A (Sigma) and 5 ng/ml IL-2 (Gibco-BRL) at 37°C for 8 h. The concanavalin A was removed by treatment with 100 μg/ml α-methyl-mannoside (Sigma) for 20 min at 37°C before rinsing in serum-free medium. The cells were resuspended at a concentration of 5×10^6 cells/ml in serum-free medium and treated with the indicated concentrations of activated recombinant matrilysin (Chemicon) for 6 h. The conditioned media were collected and clarified by centrifugation and filtration through a 0.2 μm filter (Corning) to remove cellular material.

Western analysis

Samples were subjected to SDS–PAGE in a 15% gel then electrophoretically transferred to nitrocellulose. The blots were blocked with 5% non-fat dry milk in Tris-buffered saline pH 7.4 and the primary antibodies were added in fresh blocking solution. The anti-FLAG M2 (Kodak) and anti-FasL (Transduction Laboratories) antibodies were used at a dilution of 1:1000. For the FLAG western, a horseradish peroxidase (HRP)-conjugated secondary antibody (Promega) and chemiluminescent detection (p-coumaric acid (Sigma), 200 μM; luminol (Sigma), 1.25 mM; 100 mM Tris base pH 8.5) was used to visualize the immunoreactive bands. For the FasL western, a biotinylated secondary antibody (Vector Labs) and HRP-labeled streptavidin (Jackson ImmunoResearch) were used for detection and the bands were visualized using enhanced chemiluminesence (Amersham).

Immunohistochemistry

The paraffin-embedded tissue was cut in 5 μm sections onto glass slides, deparaffinized in xylene and rehydrated. Immunohistochemical staining was performed following microwave antigen retrieval (3 min, high power in 100 mM sodium citrate pH 6.0). The primary antibodies were detected with biotinylated secondary antibodies and ABC amplification (Vector Labs). The HRP-labeled immune complex was detected using the substrate True Blue (KPL Laboratories) and counterstained with Contrast Red (KPL Laboratories). Photographs were taken using a Zeiss photomicroscope and a CCD camera. The primary antibodies used were: affinity-purified rabbit polyclonal anti-mouse matrilysin (1:5,000) [24], affinity-purified rabbit polyclonal anti-FasL C-178 (1:1,000, Santa Cruz) and affinity-purified rabbit polyclonal anti-Fas M-20 (1:1,000, Santa Cruz).

Apoptosis assays

The 293 cells were grown in DMEM (Gibco-BRL) supplemented with 10% FCS and were plated at a concentration of 1×10^6 cells per well of a six-well cell culture dish one day before conditioned medium transfer. The conditioned medium was transferred to monolayers of 293 cells from which the growth medium had been removed, and incubated for 22 h. This incubation was performed in the presence of 2 μg/ml of the FasL-neutralizing antibody, Kay-10 (Pharmingen) or of an isotype control (Pharmingen). The cells were harvested for analysis of apoptosis by centrifugation and rinsing in PBS. Cell pellets were resuspended in 300 μl of PBS to which was added 700 μl of 100% ethanol. The cells were pelleted and resuspended in PBS containing 50 μg/ml RNase A for 30 min at room temperature. Propidium iodide was then added to a final concentration of 50 μg/ml and the samples analyzed by flow cytometry using a FACStar. Data analysis was performed using the software package FlowJo (Treister Enterprises). As a positive control, cells were incubated with 0.5 μg/ml of the CH11 Fas-activating antibody (Upstate Biologicals) resulting in a threefold increase in the sub-G1 peak.

For DNA laddering analysis, monolayers of 293 cells grown on 60 mm dishes were exposed to treated or untreated splenocyte conditioned medium for 22 h and the cells harvested by scraping and centrifuging. They were washed once in PBS and lysed in laddering lysis buffer (1% NP-40, 20 mM EDTA, 50 mM Tris pH 7.5). The supernatants were RNase-treated at 37°C for 2 h and then digested with proteinase K overnight at 55°C. The DNA was precipitated with ammonium acetate and ethanol and then resuspended in Tris-EDTA pH 8.0. Samples were normalized for DNA content, run on a 1.7% agarose gel and visualized following ethidium bromide staining.

For TUNEL analysis, 5 μm sections were rehydrated and incubated with terminal deoxytransferase (TdT) (0.25 U/μl) and biotin-dUTP (0.4 nM/ml) in $1 \times$ TdT buffer (Boehringer Mannheim) for 1 h. The slides were washed and blocked with 1% goat serum and incubated with HRP-conjugated streptavidin. HRP was detected using diaminobenzidine and the slides were counterstained with methyl green. Random areas of sections from the indicated number of wild-type or matrilysin-null animals were scored for the number of apoptotic epithelial cell nuclei per 3,000 total epithelial cell nuclei without knowledge of sample identity. The two-sample *t*-test was used to determine the statistical significance of the data.

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