Neoplasia • Vol. 3, No. 6, 2001, pp. 459–468

www.nature.com/neo

Matrilysin [MMP-7] Expression Selects for Cells with Reduced Sensitivity to Apoptosis[1]

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Abstract

The matrix metalloproteinase matrilysin (MMP-7) has been demonstrated to contribute to tumor development. We have shown previously that members of the TNF family of apoptosis-inducing proteins are substrates for this enzyme, resulting in increased death pathway signaling. The goal of the current study was to reconcile the proapoptotic and tumor-promoting functions of matrilysin. In the human HBL100 and murine NMuMG cell lines that represent early stages of tumor progression and that express both Fas ligand and its receptor, exposure to matrilysin results in cell death that can be blocked by FasL neutralizing antibodies. Constitutive expression of matrilysin in these cell lines selects for cells with reduced sensitivity to Fas-mediated apoptosis as demonstrated both with a receptor-activating antibody and with *in vitro* activated splenocytes. Matrilysin-expressing cells are also significantly less sensitive to chemical inducers of apoptosis. We propose that the expression of matrilysin that has been reported at early stages in various tumor types can act to select cells with a significantly decreased chance of removal due to immune surveillance. As a result, these cells are more likely to acquire additional genetic modifications and develop further as tumors. *Neoplasia* (2001) **3,** 459–468.

Keywords: matrix metalloproteinase, Fas ligand, tumor progression, immune surveillance, drug resistance.

Introduction

Malignant transformation occurs as a stepwise progression from normal cells to a fully metastatic tumor. During this progression, a set of traits must be acquired by cancer cells to ensure their continued existence. The necessary capabilities have been described as follows: autonomous growth signaling, disregard for cell cycle checkpoints, resistance to programmed cell death, immortalization, sustained angiogenesis, and tissue invasion and metastasis [1]. The ability to resist apoptotic signals is a trait strongly selected for in tumor development as there are a number of points at which an apoptotic signaling cascade can be initiated resulting in the destruction of the tumor. These steps may include an immune response, a hypoxic environment that can occur before establishment of an adequate blood supply, and anoikis, a form of cell death known to be induced through loss of integrin-mediated matrix contacts [2]. In addition to preventing removal of the tumor, resistance to apoptosis also creates a permissive environment for genetic instability allowing tumor cells to acquire additional genetic mutations without triggering a suicide response. Thus, the multiple stages at which death signals must be overcome and the benefits likely to accrue to a resistant tumor cell suggest that acquisition of resistance to apoptosis would be a strongly selected trait.

Matrilysin (MMP-7, E.C. 3.4.24.23) is a member of a multiple enzyme family, the matrix metalloproteinases (MMPs), which have been principally associated with degradation of extracellular matrix (ECM) components [3]. As ECM-degrading enzymes, the MMPs have been linked with tumor invasion and metastasis, and this has led to the development of synthetic MMP inhibitors as anti-invasive and antimetastatic therapies [4]. Preliminary evidence from phase III clinical trials of these agents have shown them to be of little benefit to patients with advanced cancers although in patients with earlier stage disease, survival benefits have been observed [5]. This may be explained by recent evidence, which implicates MMP activity in tumor growth [6] and angiogenesis [7]. Unlike many other MMPs, which are expressed as a host stromal response to a tumor, matrilysin is produced by tumor cells themselves at an early stage [8]. This has been observed in adenomas or premalignant lesions of the intestine, stomach, prostate, breast, and ovary [9–14]. Using genetically manipulated animals, a definite role for matrilysin in early tumor development has been established. Ablation of matrilysin significantly reduced tumor formation by 67% in the multiple intestinal neoplasia (Min) mouse model of intestinal cancer [15]. Conversely, overexpression of matrilysin in the mammary glands of MMTV-*neu* mice, a mouse model of mammary

Abbreviations: CTL, cytotoxic T lymphocyte; ECM, extracellular matrix; FasL, Fas ligand; FLIP, FADD-like interleukin-1β converting enzyme inhibitory protein; MMP, matrix metalloproteinase; NK, natural killer; sFasL, soluble Fas ligand; TNF, tumor necrosis factor

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[1]This work was funded by R01 CA60867 awarded to L. M. M. and by the Vanderbilt Ingram Cancer Center grant P30 CA68485. T. V.-G. is supported by grant 99-3051 awarded by the Susan G. Komen Breast Cancer Foundation.

Received 13 March 2001; Accepted 1 August 2001.

carcinogenesis, significantly accelerated tumor development [16]. In both of these models, an oncogenic switch (*Apc* mutation in the Min mouse or *neu* expression in the MMTV-*neu* mouse) was already present; however, matrilysin expression considerably influenced the tumorigenic potential of this mutation. The mechanism by which the MMP matrilysin contributes to early tumor growth is unknown.

Previously, we have shown that two members of the TNF family of proteins, TNF-α and Fas ligand (FasL), are substrates of matrilysin in specific circumstances [17,18]. FasL is a type II membrane protein expressed by activated T cells, natural killer (NK) cells and in cells of immune-privileged tissues such as eye and testis [19]. Expression of FasL is frequently observed in a number of tumor types, including melanoma, breast, and colon [20]. Binding of FasL to the widely expressed Fas cell-surface receptor activates an apoptotic signal cascade. Although coexpression of ligand and receptor may suggest an apoptotic event, we have previously shown that this does not necessarily occur and speculate this is due to spatial constraints. Solubilization of FasL by matrilysin results in an active death-inducing protein free to interact with the receptor [18]. Here we show that the enzyme matrilysin, acting on its substrate FasL, promotes apoptosis in early tumor cells. Chronic exposure to matrilysin, however, acted as a selective pressure for apoptotic resistance. Cells selected in this manner were significantly less sensitive to both Fas-mediated and chemically induced apoptosis. Thus, we propose a model whereby matrilysin expression in precancerous cells confers a survival advantage and promotes the development of a tumor, explaining the apparent contradictory effects of matrilysin on apoptosis and tumor progression.

Materials and Methods

Cell Lines and Expression Vectors

NMuMG (CRL 1636) and HBL100 (HTB 124) cell lines, obtained from the ATCC, were maintained at 37°C, 5% CO_2 in DMEM (Gibco BRL, Long Island, NY) containing 10% FCS (Atlanta Biologicals, Morcross, GA). The NMuMG line also required 10 μg/ml insulin (Sigma, St. Louis, MO). For selection following transfection, G418 (Mediatech, Herndon, VA) was added at 800 μg/ml, which was decreased to 200 μg/ml for maintenance. Human matrilysin cDNA [21] cut with *Hin*dIII and *Xba*I was subcloned into the pRc/RSV expression vector (Invitrogen, Carlsbad, CA). HBL100 cells were transfected with this expression plasmid or empty vector using Superfect (Qiagen, Valencia, CA). NMuMG cells were infected with a retrovirus constructed by inserting the human matrilysin cDNA cut with *Eco*RI into the LXSN vector [22]. Constructs were verified by DNA sequencing.

Antibodies and Other Reagents

Antibodies used were rat monoclonal anti-matrilysin [23], rabbit anti-Fas (N-18, Santa Cruz Laboratories, Santa Cruz, CA) and anti-FasL (C-178, Santa Cruz); monoclonal anti-Fas (clone 13, Transduction laboratories, San Diego, CA); monoclonal anti-human Fas clone CH11 (Upstate Biologicals, Lake Placid, NY); monoclonals anti-mouse FasL clone Kay-10, anti-human FasL clone NOK-2, anti-human FasL clone G247-4, and monoclonal anti-mouse Fas clone JO-2 (all from Pharmingen, San Diego, CA). Appropriate isotype controls were obtained from Pharmingen. For induction of apoptosis, the CH11 and JO-2 antibodies were used at concentrations between 0.025 and 0.5 μg/ml. For neutralization studies, the NOK-2 antibody was used at 1 μg/ml and was preincubated with conditioned media at room temperature for 1 hour. Fas-Fc, the human Fas receptor fused to an immunoglobulin Fc domain (Pharmingen) was used at 3 μg/ml for immunoprecipitation.

Recombinant active human matrilysin was from Calbiochem (La Jolla, CA). Cycloheximide and staurosporine were obtained from Sigma. The cycloheximide was used at a concentration of 1 μg/ml. Staurosporine, solubilized in dimethyl sulfoxide (DMSO, Sigma) was used at the concentrations indicated for each experiment. Mitomycin C was obtained from LKT laboratories, St. Paul, MN, and was used in the concentration range of 2.5 to 50 μg/ml.

Growth and Proliferation Assays

To determine the proliferation rate for each of the vector control and matrilysin-expressing clones, thymidine incorporation was measured. Cells (3×10^5) were plated on 35-mm dishes and left to adhere overnight. Methyl-^{3}H-thymidine (NEN, Boston, MA; 1 μCi) was added to each plate and the plates incubated for 24 hours. The cells were washed, lysed in SLS buffer (1% sodium lauryl sulfate, 0.3 N NaOH), and were harvested by scraping. Aliquots of the lysate were analyzed for protein content using the BCA assay (Pierce, Rockford, IL). Four hundred microliters of lysate from each plate was placed in a scintillation vial with 10 ml of scintillation fluid (Eco-Lume, ICN, Costa Mesa, CA) and the amount of isotope present measured using a Beckman scintillation counter.

To measure clonal growth, 500 cells of each of the clones were placed on 60 mm plates in complete growth medium and incubated for 9 days with two changes of medium. At this time the medium was aspirated and the cells were fixed with cold acetic acid:methanol (1:3) for 5 minutes. The cells were then rinsed and stained with crystal violet (Sigma). The number of visible colonies on each plate was counted.

Apoptosis Assays

For flow cytometric analysis, cells were harvested and washed in PBS with 5 mM EDTA. The cells were fixed with 70% ethanol, treated with RNase A (Sigma), and stained with 50 μg/ml propidium iodide (Sigma). The cells were analyzed using a FACStar instrument (Becton Dickinson, San Jose, CA) and data were processed with Flow-Jo software (Treistar Enterprises, San Diego, CA).

For DNA laddering, cells were grown on 60 mm dishes, treated as indicated for 22 hours, and then harvested by scraping. They were washed in PBS and lysed in laddering lysis buffer [1% NP-40; 20 mM EDTA; 50 mM Tris, pH 7.5]. Supernatants were RNase-treated at 37°C for 2 hours and

digested with proteinase K overnight at 55°C. The DNA was precipitated and resuspended in Tris–EDTA, pH 8.0. Samples were normalized for DNA content, run on a 1.7% agarose gel, and visualized with ethidium bromide staining.

For Hoechst staining, cells were grown on chamber slides (Nunc). The cells were fixed in 25% (v/v) acetic acid/methanol and incubated for 15 minutes with 0.1 μg/ml bisbenzimide (Hoechst 33258, Sigma). Cells were viewed with a UV-equipped fluorescence microscope.

Protein Expression Analysis

Protein concentrations of samples were determined using the BCA assay (Pierce) and equal quantities of protein were used for Western blot analysis. Samples were electrophoresed through 12% SDS-PAGE gels and transferred to nitrocellulose. The antibodies used were rat monoclonal anti-matrilysin, mouse monoclonal anti-FasL (clone G247-4), mouse anti-Fas (clone 13), and rabbit anti-FasL (N-18) or anti-Fas (C-178). The blots were washed and incubated with biotinylated anti-species secondary antibodies (Vector Laboratories, Burlingame, CA). After washing, the blots were incubated with streptavidin–HRP (Jackson Immuno-Research, West Grove, PA). Signal was detected by chemiluminesence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ).

To analyze cell-surface expression of FasL, cell-surface proteins were labeled with biotin. The cells were washed in cold PBS plus 0.1 mM CaCl and 1 mM $MgCl_2$. The cells were incubated on ice for 30 minutes with 1.5 mg/ml Sulfo-NHS-LC-biotin (Pierce). Cells were washed with PBS plus 100 mM glycine and then with PBS containing 1% BSA. The cells were lysed in lysis buffer (0.5% NP-40; 100 mM NaCl; 50 mM Tris–HCl, pH 7.5). Equal quantities of lysates were immunoprecipitated with recombinant Fas:Fc and protein A-sepharose (Amersham Pharmacia Biotech). The protein A-sepharose beads were washed five times with lysis buffer, mixed with Laemmli sample buffer, boiled for 10 minutes, and loaded onto 12% SDS-PAGE gels. The gels were blotted onto nitrocellulose and probed with streptavidin–HRP. Bands were visualized using chemiluminesence.

For flow cytometric analysis of cell-surface protein expression, cells were harvested using 5% EDTA in PBS. A total of 2×10^5 cells per sample, suspended in 1% BSA in PBS plus 0.1% sodium azide, were incubated with primary antibody or a suitable isotype control for 1 hour on ice. Antibodies used were rabbit anti-Fas (N-18) and mouse anti-Fas (clone 13). After washing in BSA/PBS, cells were incubated with FITC-conjugated secondary antibodies (Gibco BRL) for 1 hour on ice. The cells were again washed and analyzed using a FACStar instrument. Data were processed using Flow-Jo.

T-cell–induced apoptosis spleen cell isolation was performed as described [18]. Briefly, spleens from C57/Bl6 mice were macerated and splenocytes were isolated following removal of red blood cells by hydrolysis. Activation was achieved by incubation with 10 μg/ml concanavalin A (Sigma) and 5 ng/ml IL-2 (Gibco-BRL) at 37°C for 8 hours. The concanavalin A was removed by addition of 100 μg/ml α-methyl-mannoside (Sigma) for 20 minutes at 37°C before rinsing in RPMI medium. The cells were counted and transferred to monolayers of NMuMG or HBL100 clones in the presence of serum, cycloheximide, and insulin (for NMuMGs) such that the ratio of splenocytes to epithelial cells was 5:1. After 6 hours, the monolayers were washed with PBS until no splenocytes were visible on the plates. The cells were then re-fed with DMEM containing serum, cycloheximide, and insulin. After a further 16 hours, the cells were harvested and stained with propidium iodide as above.

Results

Characterization of an In Vitro *Model System*

We hypothesized that the generation of sFasL by matrilysin could promote tumor development by acting as a selective pressure for apoptotic resistance. To test this, we examined the effects of matrilysin on epithelial cells representing early stages of tumor development that are sensitive to Fas-mediated death. The NMuMG cell line was established from normal murine mammary gland epithelium [24] and has been reported to produce benign cystadenomas [25] or to be nontumorigenic in mice [26]. HBL100 cells were originally believed to represent normal human breast epithelium [27] but, although ostensibly derived from breast milk, a quality control analysis by the ATCC revealed the presence of a Y chromosome.[1] Importantly, they are nontumorigenic when injected into nude mice and so are considered a suitable model for our studies.

NMuMG and HBL100 cells were first characterized with respect to matrilysin expression and the Fas pathway. Neither cell line expressed matrilysin as determined by western blotting of conditioned media (data not shown). However, by Western blotting of total cell lysates, expression of both the Fas receptor and its ligand FasL was observed (Figure 1*A*). To confirm that these proteins were localized to the cell surface, flow cytometric analysis or cell-surface biotinylation was performed. Cell-surface expression of ligand and receptor was detected on both cell lines using flow cytometry with two different antibodies for each antigen (Figure 1*B* and data not shown). As there have been reports that some of the antibodies to FasL may not detect this antigen specifically, we also used a cell-surface biotinylation procedure followed by immunoprecipitation with the Fas receptor binding site linked to the immunoglobulin Fc domain (Fas-Fc) to confirm cell-surface expression (Figure 1*C*). Thus, both NMuMG and HBL100 cells express Fas and FasL on their cell surfaces.

To determine whether the Fas receptor was functional in these cell lines, the cells were treated with a Fas receptor–activating antibody for 22 hours. The cells were then harvested for propidium iodide staining and flow cytometric evaluation of DNA content or for DNA laddering experiments. Apoptosis, as defined by either the presence of DNA ladders following agarose gel electrophoresis (Figure 1*D*) or a

[1]Information from ATCC: www.ATCC.org/phage/probline.html.

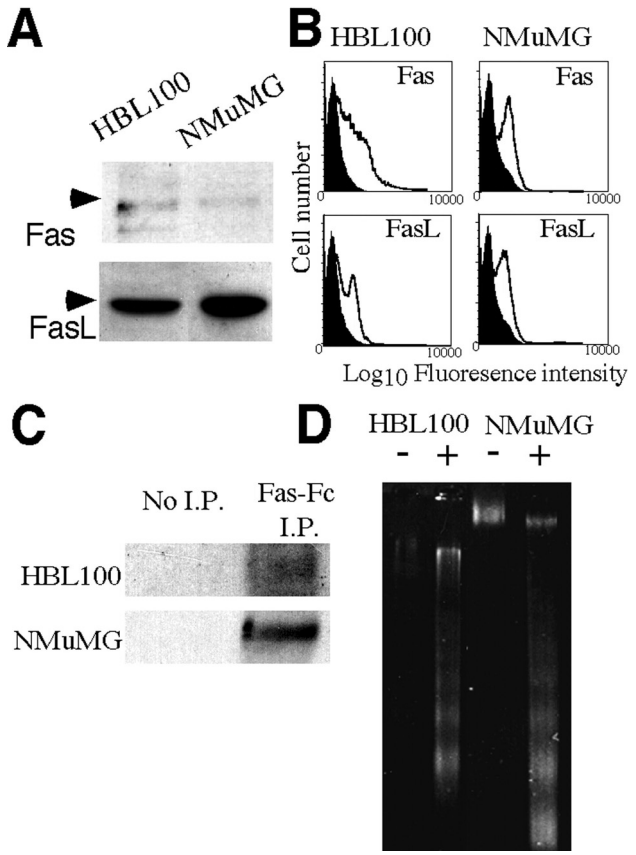

Figure 1. *Fas and FasL are expressed and the Fas pathway is functional in NMuMG and HBL00 cells. (A) Western blotting of total cell lysates from HBL100 and NMuMG cells for Fas and FasL, using the M20 anti-Fas and C178 anti-FasL antibodies, respectively. (B) Flow cytometric analysis of cell-surface expression of Fas and FasL using the clone 13 anti-Fas and clone 33 anti-FasL antibodies. The solid black peak in each graph indicates the background staining observed with a control IgG. Similar traces were obtained when the M20 anti-Fas and C178 anti-FasL antibodies were used (not shown). (C) Immunoprecipitation of biotinylated surface-associated FasL. For each cell line, lysates from biotinylated cells were subjected to the immunoprecipitation procedure in the absence (No I.P.) or presence (Fas-Fc I.P.) of Fas-Fc protein. A band of approximately 40 kDa was specifically detected in the I.P. lane for each cell line. (D) Genomic DNA was isolated from NMuMG or HBL100 cells that had been treated with the Fas-activating antibodies Jo-2 and CH11, respectively (+) or isotype control (−) and was analyzed by agarose gel electrophoresis. Cycloheximide at a concentration of 1 μg/ml was included with the NMuMG cells. The characteristic DNA laddering pattern associated with apoptosis can be clearly seen in the Fas antibody-treated lanes.*

subG1 peak in propidium iodide–stained cells analyzed by flow cytometry occurred in Fas antibody–treated cells. The NMuMG cell line required the presence of the protein synthesis inhibitor cycloheximide to potentiate the antibody response, a requirement reported previously for some cell lines [28]. The HBL100 cells, however, underwent apoptosis in response to antibody treatment alone. For the NMuMG cells the percentage of population with a subG1 content of DNA was 51.2±0.1 in the presence of the murine Fas-activating antibody plus cycloheximide compared with 4.2±0.1 for a control IgG plus cycloheximide. In the case of the HBL100 cells, the human Fas-activating antibody resulted in a subG1 percentage of 21.4±2.9 compared with 6.1±1.1 for a control IgM.

Treatment with Matrilysin Induces Apoptosis in NMuMG and HBL100 Cells

The characterization results showed that the NMuMG and the HBL100 cell lines express both Fas and FasL and that the Fas pathway is functional. To determine if matrilysin-dependent solubilization of FasL could induce cell death in the NMuMGs and HBL100s, the cells were treated with increasing concentrations of recombinant active matrilysin and the extent of apoptosis analyzed after 24 to 48 hours. Apoptosis, as measured by the percentage of DNA with a subG1 DNA content, was induced in these cell lines by matrilysin in a dose-dependent manner (Figure 2*A*). The addition of 100 ng/ml of matrilysin resulted in a three-fold increase in the apoptotic index in HBL100 cells and a two-fold increase in NMuMG cells. To distinguish between an effect on anoikis resulting from degradation of cell-produced ECM and a Fas-mediated event, clarified conditioned media from matrilysin-treated HBL100 cells was transferred to a fresh monolayer of the same cells. This was performed in the presence of serum, which contains the general proteinase inhibitor, α-macroglobulin, to inhibit any residual matrilysin activity in the transferred medium. As before, apoptosis was induced in cells exposed to conditioned medium from matrilysin-treated cells but not from untreated cells (Figure 2*B*). The addition of a FasL-neutralizing antibody (NOK-2) completely blocked the increase in apoptosis, indicating that death was indeed mediated by sFasL (Figure 2*B*). This result is similar to a previously reported experiment in which matrilysin-generated sFasL from murine splenocytes induced apoptosis in the kidney cell line, 293, that could be blocked by inclusion of a different FasL neutralizing antibody (Kay-10) [18].

Establishment of HBL100 and NMuMG Lines Constitutively Expressing Matrilysin

Previous work indicated that, following androgen withdrawal, the normal epithelial cells of the prostate gland express matrilysin and FasL and undergo apoptosis in a Fas- and matrilysin-dependent manner [18]. In contrast, studies with tumor cells indicate that early tumors (e.g., intestinal adenomas) express Fas [29], FasL [30], and matrilysin [15], and yet consistently grow larger. To reconcile this apparent discrepancy, we hypothesized that long-term exposure to matrilysin-generated sFasL selected for cells that have acquired resistance or lowered sensitivity to Fas-mediated apoptosis. To test this, matrilysin was constitutively expressed in the NMuMG and HBL100 cell lines. Variant lines of both NMuMG and HBL100 were created by stable expression of the human matrilysin gene under the control of viral promoters. Control cells were infected or transfected with the appropriate empty vector. After selection in G418, viable colonies were formed in both matrilysin-expressing as well as in vector control populations. Several clones from the four populations were selected for expansion and these clonal lines were used for further analysis. Western blotting demonstrated the presence of matrilysin in conditioned media from matrilysin-transfected clones (Figure 3*A*) whereas the vector-control clones remained

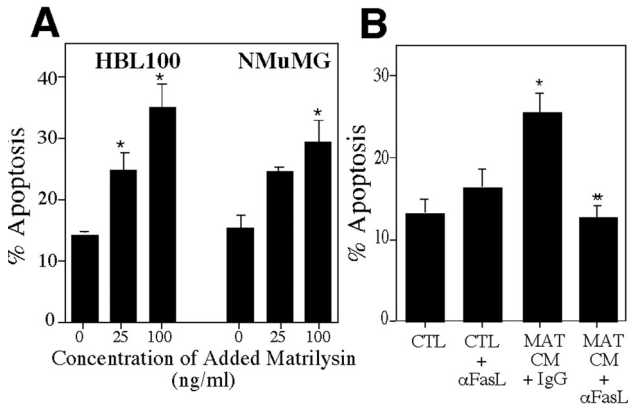

Figure 2. *Short-term treatment with matrilysin induces cell death. (A) NMuMG or HBL100 cells were treated with the indicated amounts of recombinant activated matrilysin in the presence of 1 μg/ml cycloheximide (for NMuMGs) in serum-free medium for 24 hours. The cells were harvested, stained with propidium iodide, and analyzed by flow cytometry. The percentage of cells with subG1 DNA (% apoptosis) from three separate experiments, performed in triplicate were used to calculate the mean ± standard error (SEM). Asterisks indicate significant differences (P < .05) from the untreated (0 ng/ml matrilysin) samples, calculated using Student's t test. (B) Serum-free conditioned medium was obtained from HBL100 cells treated with 0 (CTL) or 100 ng/ml matrilysin (MAT CM) for 48 hours. This CM was filtered and incubated for 1 hour at room temperature with the NOK-2 anti-FasL (αFasL) antibody or an IgG control (IgG). The medium was then transferred to fresh monolayers of HBL100 cells in the presence of an equal volume of serum-containing DMEM and incubated for 22 hours. The cells were harvested, stained with propidium iodide and analyzed by flow cytometry. The subG1 population from two separate experiments performed in triplicate was used to calculate the mean ± SEM for each condition. *Indicates a significant difference from control, P = .0035. **Indicates a significant difference from MAT CM + IgG, P = .018.*

negative. Analysis of conditioned media from these clones indicated the presence of soluble FasL (sFasL) exclusively in the matrilysin-expressing clones (Figure 3*A*). Analysis of proliferation by thymidine incorporation indicated that growth rates were not significantly altered in the matrilysin-expressing clones (Figure 3*B*). Clonal growth ability did not differ significantly from controls in three of the four matrilysin-expressing clonal variants (Figure 3*C*). One HBL100 clone (Mat1) had reduced clonal growth, and HBL100 clones were not used in subsequent experiments where growth at low density was important. Fas receptor levels were comparable in both matrilysin-expressing and the vector control clones (3D), indicating that loss of receptor was not a result of matrilysin expression.

Matrilysin-Expressing Clones Demonstrate Reduced Sensitivity to Fas-Mediated Apoptosis

As the matrilysin-expressing clones appeared to be viable despite their constant exposure to sFasL, we tested their sensitivity to the Fas pathway. The cells were treated with increasing concentrations of Fas-activating antibody in the presence or absence of cycloheximide and the extent of apoptosis 22 hours later analyzed by flow cytometric evaluation of propidium iodide-stained cells. The amount of apoptosis induced in antibody-treated matrilysin-expressing cells was reduced by approximately 50% compared to vector control cells in both the HBL100 and NMuMG clones (Figure 4*A* – *C*, *F*–*H*). These results were confirmed by Hoechst staining and counting of apoptotic nuclei (Figure 4*D*, *E*, *I* and *J*). The relative reduction in sensitivity to the apoptosis-inducing effect was retained over a concentration range of 0.025 to 0.5 μg/ml of the Fas-activating antibody (Figure 4*B* and *G*).

Matrilysin-Expressing NMuMG Clones Demonstrate Reduced Sensitivity to Drug-Induced Apoptosis

To test whether matrilysin-expressing clones were resistant to other apoptotic stimuli, the NMuMG clones were

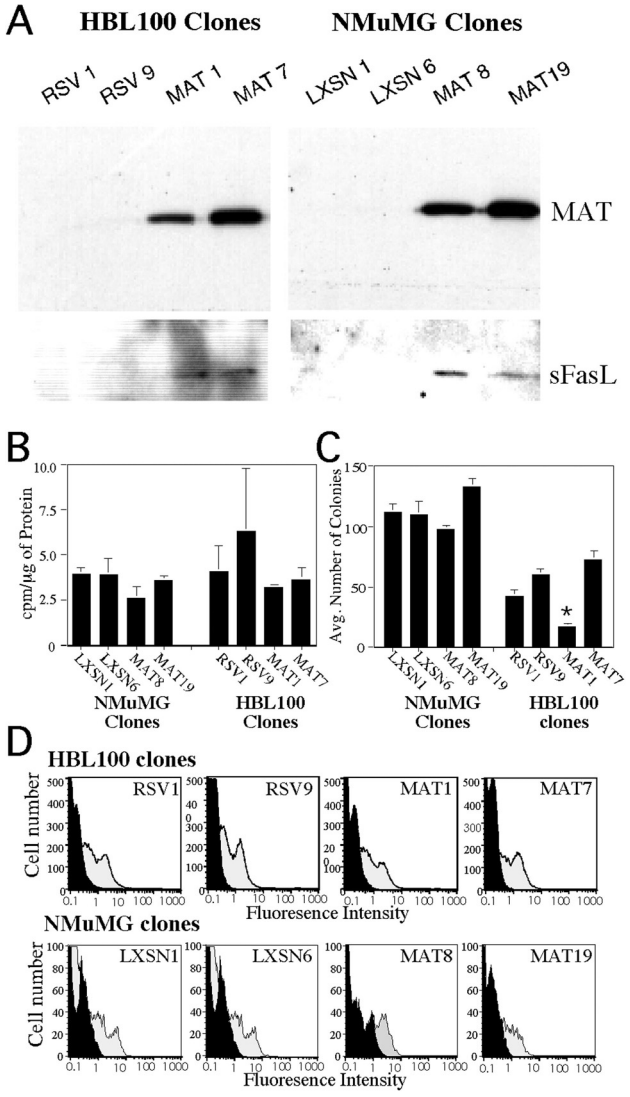

Figure 3. *Matrilysin can be expressed in Fas-expressing cells. (A). Western blotting for matrilysin ["MAT," upper panels] or sFasL [lower panels] from 48-hour conditioned media from the indicated vector (LXSN or RSV) or matrilysin-expressing (MAT) clones. The antibodies used for detection of sFasL were G-247-4 anti-human FasL and Kay-10 anti-murine FasL. (B) Measurement of tritiated thymidine incorporation in the various clones. (C) Clonal growth analysis for each of the clones. Five hundred cells were plated on plastic in growth medium and the number of visible colonies counted after 10 days. The asterisk indicates a result significantly lower than vector control cells (P < .05). (D) Cell-surface expression of the Fas receptor in each of the clones. Antibodies used were the rabbit anti-Fas polyclonal antibody or a rabbit IgG control followed by an FITC-conjugated anti-rabbit antibody. The solid black peak represents the results obtained for the rabbit IgG and the clear peak in each plot represents the result obtained for the anti-Fas antibody. Results shown are representative of two separate analyses.*

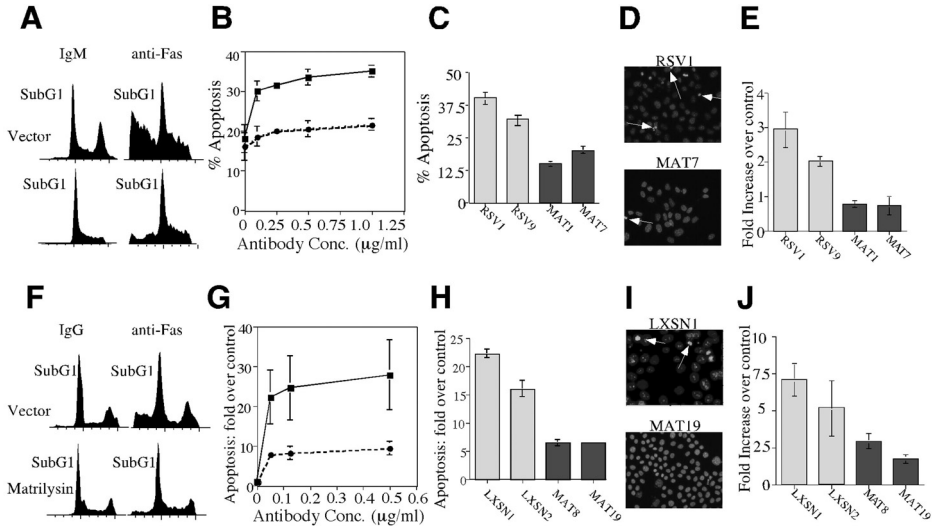

Figure 4. *Matrilysin-expressing clones demonstrate reduced sensitivity to Fas-mediated apoptosis. HBL100 (panels A–E) and NMuMG (panels F–J) cells were treated with Fas-activating antibodies for 22 hours in the absence (HBL100) or presence (NMuMG) of cycloheximide. The cells were harvested and stained with propidium iodide before analysis by flow cytometry. (A, F) Representative samples of the tracings obtained for a vector-control (HBL100, RSV; NMuMG, LXSN) and matrilysin-expressing (MAT) clone from each cell line following exposure to isotype control or Fas-activating antibodies are shown. (B, G) The vector-control and matrilysin-expressing clones from each cell line were exposed to a range of concentrations of Fas-activating antibody. The results for both vector clones (RSV or LXSN: solid line, squares) or both matrilysin-expressing clones (MAT: dashed line, circles) have been combined. (C, H) Bar graphs showing the mean and SEM obtained for the independent clones when an antibody concentration of 0.5 μg/ml was used in three independent experiments. (D, I) Sample photomicrographs showing bisbenzimide-stained cells following exposure of the various clones to 0.5 μg/ml antibody. The characteristic nuclear morphology associated with apoptosis is clearly visible (arrows). (E, J) Bar graphs represent the mean and SEM obtained for each clone following four separate experiments.*

treated with staurosporine, an inhibitor of protein kinase C, and mitomycin C, a DNA cross-linker. As described previously, flow cytometry was used to assess the extent of apoptosis as a result of drug treatment. When the NMuMG cells were treated with increasing concentrations of staurosporine (0.1 to 1.0 μM) for 20 or 40 hours, significant resistance to cell death was observed in the matrilysin-expressing clones compared to vector clones (Figure 5*A* and *B*, and data not shown). Mitomycin C induced low levels of apoptosis in NMuMG cells after 20 hours of treatment; however, a significant difference in response was evident between the vector control and matrilysin-expressing clones, with the matrilysin-expressing clones demonstrating an approximately 80% reduction in apoptosis (Figure 5*C* and *D*). The effect of these drugs on apoptosis was confirmed by Hoechst staining and identification of apoptotic nuclei (data not shown). These data indicate that matrilysin expression can select for cells with significantly reduced sensitivity to a variety of apoptotic signals.

To investigate whether Fas stimulation alone could select for the resistant phenotype, vector control and matrilysin-expressing NMuMG clones were treated with Fas-activating antibody (Jo-2) or IgG at 0.25 μg/ml in the presence of cycloheximide for 22 hours. The medium was then changed

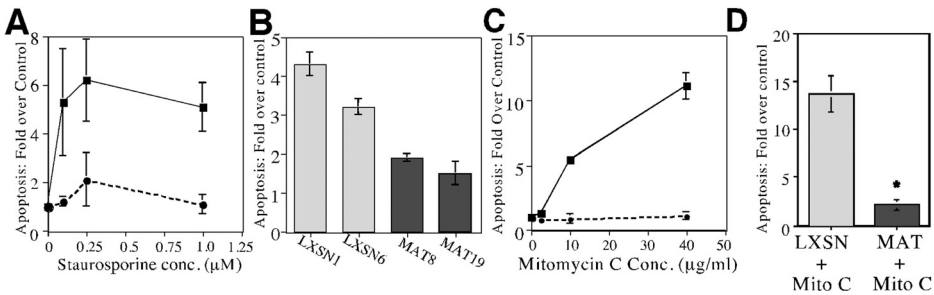

Figure 5. *Matrilysin-expressing NMuMG clones are significantly less sensitive to drug-induced apoptosis. (A) Vector control (LXSN: solid black line, squares) or matrilysin-expressing (MAT: dashed line, circles) clones were treated with a range of concentrations of staurosporine (0.1 to 1.0 μM) or an equivalent amount of vehicle (DMSO) for 40 hours. The cells were then harvested, stained, and analyzed as before. The results for the two vector clones as well as for the two matrilysin-expressing clones have been combined. (B) The bar graph indicates the mean and SEM obtained for each independent clone at a staurosporine concentration of 1 μM from three experiments. Results shown are fold over control where the control was treatment with an equivalent amount of vehicle (DMSO). (C) Vector (LXSN) and matrilysin (MAT) clones were treated for 20 hours with mitomycin C over a concentration range of 2.5 to 40 μg/ml. Following treatment the cells were harvested, stained with propidium iodide, and analyzed by flow cytometry. The results obtained for two different vector and matrilysin-expressing clones were combined. The results shown are fold over control where the control was treatment with an equivalent amount of vehicle (sterile water). (D) The bar graph shows the results obtained for one concentration of mitomycin C (50 μg/ml) after 22 hours of treatment. *Statistical significance was determined using Student's t test ($P \leq .002$).*

and the cells maintained in low levels of the activating antibody or IgG (0.025 μg/ml) without cycloheximide for 9 days to allow outgrowth of surviving cells. The cells were then exposed either to a second high dose of antibody in the presence of cycloheximide or to mitomycin C. Vector control cells that had been exposed only to IgG died as expected in response to either anti-Fas (Figure 6*A*) or mitomycin C (Figure 6*B*). In contrast, vector control cells that had been selected in Fas-activating antibody demonstrated a resistance phenotype to both stimuli similar to that seen with matrilysin-expressing clones. The matrilysin-expressing clones behaved similarly, irrespective of whether they had been selected with IgG or anti-Fas. These results reinforce our overall hypothesis that exposure to a Fas-activating signal, such as matrilysin-generated sFasL or the agonistic antibody, selects for cells with reduced sensitivity to apoptosis.

Matrilysin-Expressing Cells Are Less Sensitive to Immune-Mediated Killing

One of the earliest challenges to tumor establishment *in vivo* is the host immune response. This is dependent on a number of immune mechanisms, both innate and acquired [31]. Among the best studied responses is the generation of NK and cytotoxic T lymphocytes (CTLs) that can kill tumor cells through two pathways, a perforin/granzyme pathway or a FasL-dependent mechanism [30]. We therefore tested if the observed decrease in sensitivity to apoptosis could translate to a possible immune evasion mechanism. Murine spleen cells were isolated and activated with concanavalin A and interleukin-2 to upregulate FasL. The murine splenocytes were incubated with monolayers of the allogeneic NMuMG clones at a ratio of 5:1 for 6 hours. After this time, the splenic lymphocytes were removed and the monolayers rinsed five times before incubating overnight. The following day, the cells were harvested, stained with propidium iodide, and analyzed by flow cytometry for extent of apoptosis. The vector control clones showed an apoptotic rate of approximately 45% whereas the apoptotic rate of the matrilysin-expressing clones was 20%, a greater than 50% reduction in sensitivity (Figure 7). When murine splenocytes were incubated with the xenogeneic HBL100 clones, a similar trend was observed in that the percentage of apoptotic matrilysin-expressing cells was only 27% compared to 43% for the vector control clones (Figure 7). Thus, cells chronically exposed to matrilysin demonstrated reduced sensitivity to challenge by activated lymphocytes.

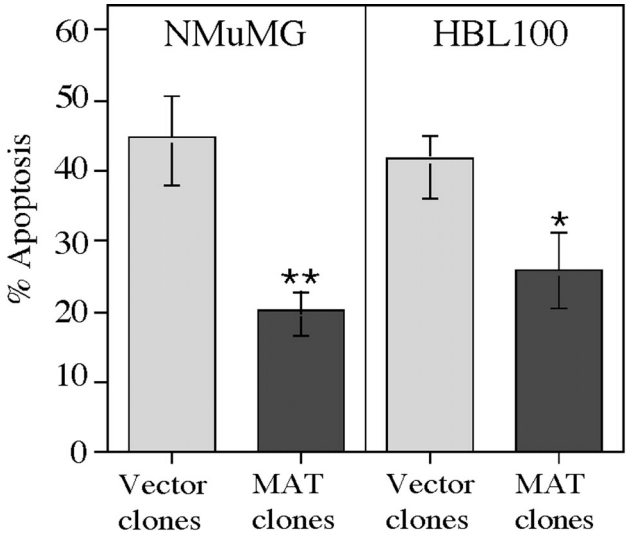

Figure 7. *Matrilysin-expressing clones demonstrate reduced sensitivity to immune cells. Monolayers of each of the vector control (Vector) or matrilysin-expressing (MAT) clones were incubated for 6 hours with freshly isolated, in vitro activated splenocytes in the presence of 1 μg/ml cycloheximide. The monolayers were then washed extensively to remove the spleen cells and the clones were further incubated for 16 hours, again in the presence of cycloheximide. The cells were harvested, stained with propidium iodide and the extent of apoptosis (subG1 DNA) determined by flow cytometry. Results shown are mean and SEM from three separate experiments. For each cell line, the results obtained for two different vector and two matrilysin-expressing clones were combined. Statistical significance was determined using Student's t test and the results are indicated with asterisks where ** represents P = .0014 and * represents P ≤ .03.*

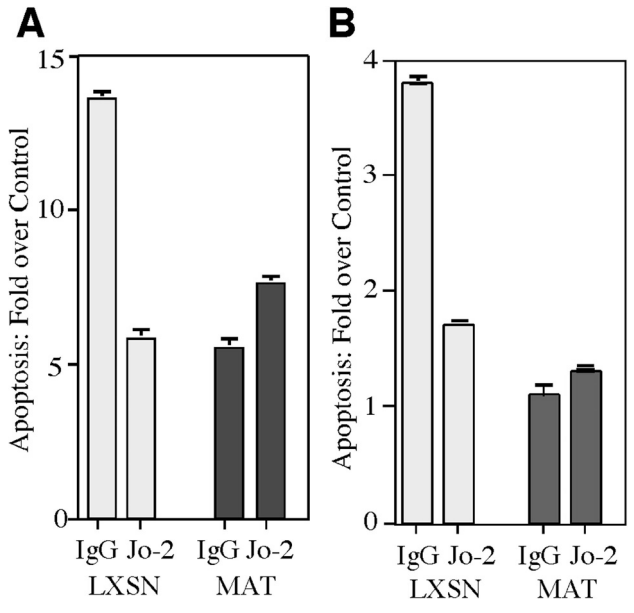

Figure 6. *Exposure to the Fas-activating antibody selects for cells with reduced apoptotic sensitivity. Vector control (LXSN) and matrilysin-expressing (MAT) NMuMG clones were treated for 22 hours with 0.125 μg/ml anti-Fas antibody (Jo-2) or isotype control (IgG) in the presence of 1 μg/ml cycloheximide. The medium was then aspirated from the remaining plates and the cells re-fed with growth medium containing 0.025 mg/ml Jo-2 or IgG. After 9 days, the cells were again treated for 22 hours with 0.125 μg/ml anti-Fas antibody in the presence of 1 μg/ml cycloheximide (A) or with 50 μg/ml mitomycin C (B). At this point, the cells were harvested, stained with propidium iodide and analyzed by flow. Results are mean and SEM from two separate experiments.*

Discussion

The MMP matrilysin has been demonstrated to contribute to early stages of mammary and intestinal tumor progression [15,16]. The substrates responsible for the tumor-promoting activity of matrilysin have not been identified. Reported substrates that could conceivably influence tumor progression include β4-integrin [32], E-cadherin [33], or the

apoptosis-related proteins TNF-α [17] and FasL [18]. In previous work, we have shown that involution of a normal prostate gland following androgen withdrawal is greatly influenced by matrilysin and associated with induction of prostatic epithelial cell apoptosis by matrilysin-generated sFasL [18]. Many tumors are known to upregulate FasL expression. Interestingly, in breast cancer specimens, matrilysin and FasL may colocalize, as both have been described as being expressed in normal-appearing epithelium adjacent to tumor areas [13,34]. However, the possibility that FasL was a relevant substrate for matrilysin in early-stage mammary or intestinal tumorigenesis presented a conundrum, as increased apoptosis should result in suppression rather than enhancement of tumorigenicity. It has become increasingly clear that tumor cells develop mechanisms to escape from the protective effects of programmed cell death. Thus, we hypothesized that chronic expression of matrilysin in the cellular precursors to breast or colon cancer could result in the selection of a population of cells with reduced sensitivity to apoptotic death. The results presented in this work support this hypothesis. Constitutive expression of matrilysin in epithelial cells that are sensitive to apoptosis induced by matrilysin cleavage of FasL resulted in at least a 50% reduction in the response of these cells to both Fas-mediated and drug-induced cell death. Thus, we speculate that these cells are more likely to survive additional insults, accumulate genetic mutations, and convert to malignancy. In particular, we report that these cells are more likely to survive lymphocyte-mediated immune surveillance mechanisms.

The activity of sFasL is a controversial topic. A number of studies have shown that overexpression of a human sFasL species equivalent to a naturally occurring form resulted in limited cytotoxic activity, whereas a murine sFasL had no cytotoxic activity [35,36]. The majority of these studies have used Jurkat T cells or other cells of lymphoid origin as targets. We have previously shown, using an epithelial target cell line, that matrilysin-generated murine sFasL does have cytotoxic activity [18]. Thus, we have proposed that the nature of the target may affect its ability to be killed by sFasL. A recent report shows that the presence of an accessory molecule such as ICAM-1 on the target cell can influence the cytotoxicity of FasL [37]. It is possible that other accessory molecules on epithelial cells may potentiate the cytotoxic activity of sFasL. Data presented in this paper illustrate that acute exposure of FasL-expressing cells to matrilysin results in the generation of a soluble factor whose activity can be abrogated by incubation with a FasL neutralizing antibody, thus suggesting that the factor is sFasL. However, we cannot completely rule out the possibility that other TNF-related molecules could be processed to a soluble form by matrilysin and that anti-FasL antibodies may cross-react with these molecules.

In addition to reports of sFasL being noncytotoxic, sFasL has also been suggested to act antagonistically to the membrane form of FasL [35]. That is, the soluble form occupies receptors without inducing a cell-death cascade while simultaneously blocking those receptors from access by cytotoxic FasL. This is however, contrary to our observation that parental cells expressing Fas and FasL do not undergo apoptosis constitutively, whereas short-term treatment with matrilysin resulting in the generation of a soluble factor does initiate FasL-dependent cell death. The generation of resistance in vector control cells following repeated exposure to the Fas-activating antibody also suggests that the mechanism of resistance is not through blockade of the receptor by a nonsignaling soluble form of the ligand. Finally, the cross-resistance to nonreceptor stimuli such as staurosporine lends further support to loss of sensitivity being due to something other than an antagonistic form of FasL. We therefore, conclude that matrilysin releases a proapoptotic form of sFasL, an observation that does not rule out the possibility of inhibitory forms of sFasL being generated by other enzymes.

Desensitization to Fas ligation following selection of apoptosis-resistant cells through exposure to chemotherapeutic agents has been described by a number of investigators. This was initially thought to be a result of these chemotherapeutic agents acting through the Fas/FasL pathway. Recent evidence, however, indicates that the majority of these agents do not in fact depend on the Fas/FasL interaction for efficacy [38,39] and the consensus now appears to be that only 5-fluorouracil has the potential to involve Fas/FasL [40,41]. A recent paper describes how matrilysin expression in tumor lines results in loss of responsiveness to chemotherapeutic agents [42]. The results are consistent with our findings with respect to drug-induced apoptosis. However, the authors suggest that the agents used exert their effects through FasL/Fas interactions and that matrilysin activity removes FasL from the cell surface, thus preventing it from participating in the death signaling pathway. Our data indicating that matrilysin-generated sFasL can initiate apoptosis contradicts the conclusions reached by these authors. We have deliberately chosen chemical agents that are not known to act through the Fas pathway and have still demonstrated that matrilysin expression leads to a loss of sensitivity. Our conclusion, therefore, is that matrilysin expression, through generation of a chronic Fas-activating signal, results in an alteration of the apoptosis signaling pathway leading to a loss of sensitivity to various death stimuli. The demonstration that cells can be selected for resistance to drug-induced cell death following incubation with the Fas-activating antibody supports this theory.

The theory of immune surveillance contributing to the eradication of tumor cells has been developed over many years [43]. Much work has gone into identifying the cell types responsible for this function of the immune system. A competent immune system, principally CD8+ CTLs, has been shown to act as a tumor suppressor in a study of chemically induced tumors [44]. The Fas/FasL system has been shown to play a role in immune surveillance and deletion of tumor cells. Both NKs and CTLs can act to lyse target cells through perforin/granzyme and Fas-mediated pathways [45]. Evidence for the importance of the Fas pathway has come from a number of different studies

[46,47]. *In vivo* dependence of CTLs on the FasL pathway has been shown using tumor cells overexpressing the Fas pathway inhibitory protein, cFLIP [48]. Multiple reports have shown that downregulation or mutation of the Fas receptor occurs in a number of tumor types [49–52], and this is thought to contribute to their ability to evade immune-mediated deletion. Testing of the ability of tumor cells expressing membrane-associated FasL to escape immune surveillance has been hampered by the proinflammatory response such tumor cells invoke. However, sFasL has also been proposed as a mediator of the inflammatory response. Although there have been suggestions that sFasL is chemotactic for neutrophils [53], Hohlbaum et al. [54] have shown that, *in vivo*, it is the membrane form of FasL that results in neutrophil infiltration, whereas cells making a soluble form do not generate this inflammatory response. In fact, the soluble form can exert a protective anti-inflammatory effect. Cells expressing a noncleavable membrane form of FasL failed to form tumors, whereas cells expressing sFasL efficiently formed tumors when injected subcutaneously into nude mice [54]. Tumor cells able to generate sFasL therefore have an additional advantage in that they do not promote a strong immune infiltration. This suggests a model whereby matrilysin-expressing tumor cells generate sFasL, thus downregulating a potential inflammatory response and neutrophil infiltration while simultaneously selecting for a FasL-resistant population of tumor cells. Thus, any immune infiltration of the tumor that does occur is ineffective so allowing the tumor to continue developing, an observation supported *in vitro* by the matrilysin-expressing clones being resistant to activated splenocytes.

Another way in which the Fas/FasL system can play a role in tumor development is through the "Fas counterattack." The expression of FasL by tumor cells can result in the killing of Fas-expressing immune cells produced as a host response to the tumor [55]. Although sometimes disputed [56], this has been reported to occur in tumors of the skin, intestine, liver, esophagus, and breast [34,57–60]. It is possible that a cleavage product of FasL released from a matrilysin-expressing tumor could diffuse from the tumor and result in the destruction of immune cells around it.

We have demonstrated that chronic exposure to matrilysin in premalignant epithelial cells that express Fas and FasL can result in the selection of a subpopulation of cells that display a decreased sensitivity to the death-inducing effects of sFasL. The Fas/FasL system is one of the mechanisms used by the immune surveillance system to eliminate aberrant cells with the potential to become cancerous. Because clonal selection of cells with robust proliferative and survival properties is necessary for the progression and continued development of a tumor, we propose that the cleavage of FasL and development of resistant subpopulations is a mechanism by which matrilysin contributes to early stages of tumor progression. Additionally, such cells also demonstrate a significantly reduced sensitivity to drugs that stimulate apoptosis, suggesting a further benefit to a developing tumor that allows it to escape the effects of toxic therapies.

Acknowledgements

The authors thank Dr. John MacDougall for making the pRc/RSV matrilysin expression vector, and Dr. Kathleen Heppner Goss for preparing the LXSN-matrilysin construct. We are grateful to Dr. Deb Mays and Matt Westfall for help with the flow cytometer.

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