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Stromelysin-3 Suppresses Tumor Cell Apoptosis in a Murine Model

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Abstract Stromelysin-3 (STR-3) is a matrix metalloproteinase with a unique pattern of expression and substrate specificity. During embryogenesis and remodeling of normal adult tissues, STR-3 is produced by stromal cells in direct contact with epithelial cells undergoing regional apoptosis and selective cell survival. STR-3 is also overexpressed by interdigitating stromal cells in primary epithelial malignancies. Although STR-3 does not degrade classic extracellular matrix components, the enzyme promotes the establishment of local tumors in nude mice by as yet undefined mechanisms. STR-3 is induced when malignant epithelial cells come into contact with surrounding stromal elements; the active stromal cell-derived 45 kDa enzyme is subsequently processed to a 35 kDa protein without enzymatic activity. We have generated MCF-7 transfectants expressing wild type or catalytically inactive 45 kDa STR-3 ($STR\text{-}3_{wt}$ and $STR\text{-}3_{cat\text{-}}$) or secreted 35 kDa STR-3 (35 kDa $STR\text{-}3_{sec}$) and evaluated their implantation and survival in nude mice. Tumors developed significantly more rapidly in animals receiving $STR\text{-}3_{wt}$, rather than vector-only, $STR\text{-}3_{cat\text{-}}$ or 35 kDa $STR\text{-}3_{sec}$ transfectants. Most importantly, $STR\text{-}3_{wt}$ tumors had a significantly lower percentage of apoptotic cells than tumors derived from vector-only, $STR\text{-}3_{cat\text{-}}$ or 35 kDa $STR\text{-}3_{sec}$ transfectants. Taken together, these studies suggest that the active STR-3 enzyme may increase tumor take by suppressing tumor cell apoptosis and that 45 kDa to 35 kDa STR-3 processing limits STR-3 activity at the tumor/stromal interface. Because STR-3 is secreted as an active enzyme rather than a proform, subsequent 45 kDa to 35 kDa STR-3 processing may represent a novel mechanism for regulating enzymatic activity. J. Cell. Biochem. 82: 549–555, 2001.

Key words: matrix metalloproteinase; tumor incidence; processing; stroma

Stromelysin-3 (STR-3) is a matrix metalloproteinase (MMP) with a unique pattern of expression and substrate specificity [Basset et al., 1990; Pei et al., 1994]. Unlike many other MMPs, STR-3 does not degrade classic extracellular matrix components [Pei et al., 1994]. The only known substrates for the human STR-3 enzyme are insulin growth factor binding protein-1 (IGFBP-1) and the serine protease inhibitors, α1-proteinase inhibitor (α1-PI, α1-antitrypsin) and α2-antiplasmin [Pei et al., 1994; Manes et al., 1997]. STR-3 also differs from other MMPs that are secreted as inactive zymogens. The STR-3 "pro" domain contains an additional recognition site for the golgi-associated pro-protein convertase, furin [Pei and Weiss, 1995]. Consequently, ~60 kDa STR-3 proenzyme is processed within the constitutive secretory pathway and released as ~45 kDa active enzyme [Pei and Weiss, 1995; Santavicca et al., 1996].

In humans, rodents and amphibians, STR-3 is expressed during embryogenesis and the remodeling of normal adult tissues [Lefebvre et al., 1992; Rodgers et al., 1994; Lefebvre et al., 1995; Patterton et al., 1995; Ishizuya-Oka et al., 2000]. In these settings, STR-3 is produced by stromal cells in direct contact with epithelial cells undergoing regional apoptosis and selective cell survival.

STR-3 is also overexpressed by a variety of primary epithelial malignancies [Rouyer et al., 1995]. In our own studies, virtually all

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newly-diagnosed primary non-small cell lung cancers (NSCLC) expressed significantly higher levels of STR-3 than adjacent normal lung specimens [Anderson et al., 1995]. In these epithelial malignancies, STR-3 is synthesized by interdigitating stromal cells [Wolf et al., 1992; Muller et al., 1993; Wolf et al., 1993; Anderson et al., 1995; Porte et al., 1995; Rouyer et al., 1995]. STR-3 is also produced by stromal elements in in situ carcinomas and precursor lesions and linked with the grade and local invasiveness of early stage lesions [Kawami et al., 1993; Muller et al., 1993; Bolon et al., 1996; Santavicca et al., 1996].

The near-uniform expression of STR-3 by early stage tumors suggests that the enzyme may participate in the initial development of these malignancies. Consistent with this hypothesis, STR-3 was recently shown to promote the establishment of local tumors in nude mice [Noel et al., 1996; Masson et al., 1998; Noel et al., 2000]. However, the mechanism by which STR-3 promotes tumor take remains undefined.

To characterize STR-3 induction and bioactivity in an in vitro system, we recently developed a tumor/"stroma" coculture assay in which NSCLC cells are grown on confluent monolayers of normal pulmonary fibroblasts [Mari et al., 1998]. In these tumor/stroma cocultures, NSCLC cells induce the stromal cell secretion of the active 45 kDa STR-3 enzyme. Forty-five kDa STR-3 is then processed via a bFGF- and MMP-dependent mechanism to a major 35 kDa protein which lacks enzymatic activity [Mari et al., 1998]. Because ~60 kDa STR-3 proenzyme is processed within the constitutive secretory pathway and released as a ~45 kDa active enzyme [Pei and Weiss, 1995; Santavicca et al., 1996], the conversion of 45 kDa STR-3 to the inactive 35 kDa protein may represent a unique mechanism for limiting STR-3 activity at the tumor/stromal interface.

In this manuscript, we analyze the effects of active and catalytically inactive 45 kDa STR-3 and the 35 kDa processed protein on tumor cell implantation and survival in a murine model. These studies provide the first evidence that STR-3 may promote tumor take via a tightly regulated effect on apoptosis. In addition, these studies demonstrate that 45 kDa to 35 kDa STR-3 processing represents a novel mechanism for tightly regulating the bioactivity of this unique MMP.

MATERIALS AND METHODS

Cell Culture

The human breast carcinoma cell line, MCF-7 [Schiemann et al., 1998; Shao et al., 2000], was grown in Dulbecco's Modified Eagles Medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (FCS), 2 mmol/L L-glutamine and 50 μg/ml penicillin/streptomycin in humidified air with 5% CO_2 at 37°C.

Generation of STR-3_{wt}, STR-3_{cat-} and 35 kDa STR-3_{sec} Constructs

The wild-type STR-3 cDNA was initially subcloned into the pUC18 plasmid using vector polylinker 5′ HindIII and 3′ XbaI sites. Thereafter, a catalytically-inactive form of STR-3 (STR-3_{cat-}) was generated by replacing Glu 216 from the HEXXH signature motif with Val, using site-directed mutagenesis (Fig. 1) (Transformer site-Directed Mutagenesis Kit, Clontech, Palo Alto, CA). Mutagenic and selection oligonucleotide primers were simultaneously annealed to the pUC-STR-3_{wt} plasmid and site-directed mutagenesis was performed according to the manufacturer's instructions. The mutagenic primer, which corresponds to bp 644–668 (5′ TGGCAGCCCATGTATTTGGCCACGT 3′) of the STR-3 cDNA, introduced a single base change (bp 656, A to T). The selection primer, which converted a 3′ pUC18 SspI restriction site into an EcoRV site (5′ CTTCCTTTTCGATATCATTGAAGCATTT 3′), allowed selection of the mutated plasmid. The resulting STR-3_{cat-} construct was then sequenced and subcloned into pRc/CMV for use in transfection assays.

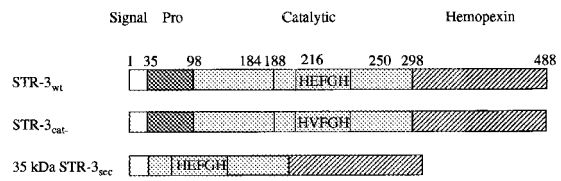

Fig. 1. Generation of transfectants expressing STR-3_{wt}, STR-3_{cat-} or 35 kDa STR-3_{sec}. Structure of STR-3_{wt} and the STR-3_{cat-} and 35 kDa STR-3_{sec} mutants. STR-3_{wt} contains a signal peptide, prodomain, catalytic domain with a HEXXH signature motif and a hemopexin domain. STR-3_{cat-} was generated by replacing Glu216 from the HEXXH signature motif with Val, using site-directed mutagenesis. Thirty-five kDa STR-3_{sec} was generated by deleting the internal sequence between the STR-3 signal peptide (aa 1–34) and the putative 35 kDa STR-3 N-terminus (aa 189) by site-directed mutagenesis.

To generate a secreted version of 35 kDa STR-3 (35 kD STR-3_{sec}), the internal sequence between the STR-3 signal peptide (aa 1–34, bp 10–111) and the putative 35 kDa STR-3 N-terminus (aa 189, bp 574) was deleted by site-directed mutagenesis (Clontech) (Fig. 1). A deletion primer which omits bp 112–573 (5′ CGCTGCTGGCCCGGGCTCTGGGGGATGTC-CACTTCGACTATGA 3′) and the above-mentioned selection primer were utilized. The resulting 35 kDa STR-3_{sec} construct was then sequenced and subcloned into pRc/CMV for use in transfection assays.

Generation and Analysis of STR-3_{wt}, STR-3_{cat-} and 35 kDa STR-3_{sec} Transfectants

pRc/CMV STR-3_{wt}, pRc/CMV STR-3_{cat-}, pRc/CMV 35 kDa STR-3_{sec} and vector-only were transfected into MCF-7 cells (2×10^6 cells per transfection) by electroporation (400 V, 125 mF). Thereafter, transfectants were selected in G 418-containing DMEM (0.8 mg/ml) and subcloned by limiting dilution. Aliquots of serum-free conditioned media from individual STR-3_{wt}, STR-3_{cat-} and 35 kDa STR-3_{sec} transfectants were analyzed for STR-3 expression by western blot as previously described [Mari et al., 1998]. Blots were incubated with a STR-3 monoclonal antibody, 1G4, which was generated against a STR-3 GST fusion protein. Three independently-derived subclones of each type of transfectant (STR-3_{wt}, STR-3_{cat-}, 35 kDa STR-3_{sec} and vector-only) were selected for further analysis.

α1-PI Degradation Assay

The enzymatic activities of STR-3_{wt}, STR-3_{cat-} and 35 kDa STR-3_{sec} were evaluated using the α1-PI assay as previously described [Mari et al., 1998] with minor modifications. In brief, 5×10^5 cells from each of the indicated transfectants were plated in 6-well plates in DMEM/10% FCS and grown until almost confluent. Thereafter, the monolayers were washed in serum-free DMEM and incubated with 1.5 ml of serum-free DMEM supplemented with 6 μg of α1-PI for 24 h at 37°C. The samples of α1-PI containing conditioned media were then harvested, concentrated 15× by ultrafiltration (Centricon 10, Amicon, Beverly, MA), and analyzed by SDS–PAGE under reducing conditions. Intact and cleaved α1-PI proteins were visualized by staining with Coomassie blue (Coomassie Brilliant Blue R250, Sigma).

Cell Proliferation Assay

The proliferation rates of STR-3_{wt}, STR-3_{cat-} and 35 kDa STR-3_{sec}, and vector-only transfectants were analyzed by MTS assay (Cell Titer 96^R Aqueous Non-radioactive Cell Proliferation Assay, Promega, Madison, MI). In brief, triplicate samples of each transfectant were plated at a density of 2.5×10^3 cells per well in 96-well plates and maintained for 48 h in DMEM/10% FCS. Thereafter, 20 μl of MTS solution was added to each well for 1 h. Reactions were then stopped with 10% SDS stop solution and optical densities determined using a microtiter plate reader at 490 nm.

Tumorigenicity Assays

Subconfluent cultures of 3 independently-derived subclones of STR-3_{wt}, STR-3_{cat-}, 35 kDa STR-3_{sec} and vector-only transfectants were trypsinized, washed, and resuspended in serum-free DMEM at 2.5×10^5 cells/ml. Cells were subsequently admixed with an equal volume of cold Matrigel (10 mg/ml, Becton Dickinson Labware, Bedford, MA). Thereafter, 5×10^4 cells were injected SC into the flanks of 6–8 week old NCr female nude mice (Taconic, NY) which had previously been implanted with 60-day release 17β-estradiol pellets (Innovative Research of America, Sarasota, FL) [Noel et al., 1996]. Ten animals were injected with each subclone.

On Days 8–20 post injection, mice were examined daily for tumor formation by two independent investigators; thereafter, mice were examined every 2 days. Tumor volumes were calculated as $0.4 \times \text{length} \times \text{width}^2$. Mice were determined to have tumors on the first day when a solid tumor of ≥ 25 mm^3 was identified. The tumor incidences in mice injected with STR-3_{wt}, STR-3_{cat-}, 35 kDa STR-3_{sec} and vector-only transfectants were compared using the log-rank test.

Apoptosis Assay

Representative tumors were harvested from three animals in each cohort on Day 24. Thereafter, tumors were fixed for 18–24 h in 4% paraformaldehyde and processed into paraffin; 3-micrometer paraffin sections were then cut and dewaxed for an analysis of apoptosis. Representative tissue sections from tumor specimens were evaluated for morphological evidence of apoptotic bodies. In addition, tissue

sections were analyzed for DNA fragmentation using TUNEL assays. Peroxidase-digoxigenin nucleotide labeling of 3′-OH DNA ends was performed according to the manufacturer's instructions (Apoptag, Oncor) with a 30 min Proteinase K incubation. Specimens were analyzed by brightfield microscopy to visualize nuclei with both TUNEL-positive staining and characteristic apoptotic body formation as previously described [Willett et al., 1999]. TUNEL-positive cells were counted on three random 100X photomicrographs of each slide. Random views were chosen by photographing fields of viable tumor cells located at 2, 6, and 10 o'clock in the tumor section, including one field on the edge of the tumor (2 o'clock), one intermediate in location (6 o'clock) and one more centrally located (10 o'clock). For each photograph, the number of darkly-stained nuclei and the total number of nuclei were counted. The results were expressed as the percentage of nuclei positive for TUNEL staining. The percentages of apoptotic cells in STR-3_{wt}, STR-3_{cat-} and 35 kDa STR-3_{sec} and vector-only tumors were compared using a paired student's *t*-test.

RESULTS

Generation and Analysis of MCF-7 Transfectants Expressing STR-3_{wt}, STR-3_{cat-} or 35 kDa STR-3_{sec}

In order to evaluate the biological activity of STR-3 in a murine tumor-bearing model, the human breast carcinoma cell line, MCF-7, was transfected with constructs encoding either wild type STR-3, catalytically inactive STR-3 or a secreted version of the processed enzyme, 35 kDa STR-3 or vector-only (Fig. 1). Three independently-derived subclones of each type transfectant (STR-3_{wt}, STR-3_{cat-}, 35 kDa STR-3_{sec} and vector-only) were selected for further analyses.

Thereafter, aliquots of serum-free conditioned media (CM) from the individual transfectants were analyzed for STR-3 secretion by western blot (Fig. 2A). As indicated, vector-only MCF-7 transfectants secreted no detectable STR-3 (Fig. 2A, lanes 1–3). In contrast, CM from STR-3_{wt} transfectants contained ~60 kDa proform, the active 45 kDa enzyme and the previously-described ~35 kDa processed protein (Fig. 2A, lanes 4–6). CM from STR-3_{cat-} transfectants secreted the proform and ~45 kDa protein; however, there was no evidence of STR-3 processing to the 35 kDa protein (Fig. 2A, lanes 7–9).

Thirty-five kDa STR-3_{sec} transfectants secreted the anticipated ~35 kDa protein (Fig. 2A, lanes 10–12). In addition, CM from these transfectants contained a higher molecular weight (MW) protein which reacted with the STR-3 antibody. This higher MW protein, which was purified and subjected to amino acid sequencing, contained only 35 kDa STR-3 amino acid sequence (data not shown), suggesting that it represents a dimer of 35 kDa STR-3.

To compare the enzymatic activities of STR-3_{wt}, STR-3_{cat-} and 35 kDa STR-3_{sec}, these proteins were tested for their ability to hydrolyze α1-PI. As expected, serum-free CM from vector-only transfectants did not cleave intact α1-PI (Fig. 2B, compare lane 1 and lanes 2, 3, 4). In contrast, wild type STR-3 efficiently cleaved α I-PI (Fig. 2B, lanes 5–7) whereas neither STR-3_{cat-} nor 35 kDa STR-3_{sec} hydrolyzed this substrate (Fig. 2B, lanes 8–10 and 11–13).

After confirming that STR-3_{wt} transfectants secreted active enzyme and STR-3_{cat-} and 35 kDa STR-3_{sec} transfectants produced catalytically inactive STR-3, the panel of vector-only, STR-3_{wt}, STR-3_{cat-} and 35 kDa STR-3_{sec} transfectants was evaluated for proliferative activity in vitro. All of the STR-3 and vector-only MCF-7 transfectants had comparable proliferation rates as assessed by MTS assay (data not shown).

Wild-Type STR-3 Increases Tumor Incidence in a Murine Model

To assess the tumorigenicity of STR-3_{wt}, STR-3_{cat-} and 35 kDa STR-3_{sec} or vector-only transfectants, three independently-derived subclones of each type were injected into the flanks of nude mice. Thirty animals were injected with each transfectant; ten animals received each of the independently-derived subclones. Following injection, the animals were closely monitored for the development of local tumors.

As indicated in Figure 3, there was a significantly higher incidence of tumor development in animals injected with STR-3_{wt}, rather than vector-only transfectants (STR-3_{wt} vs. vector-only, $P = 0.004$, log-rank test). Animals injected with STR-3_{wt} transfectants also had a significantly higher tumor incidence than those injected with either STR-3_{cat-} or 35 kDa STR-3_{sec} transfectants (STR-3_{wt} vs. STR-3_{cat-},

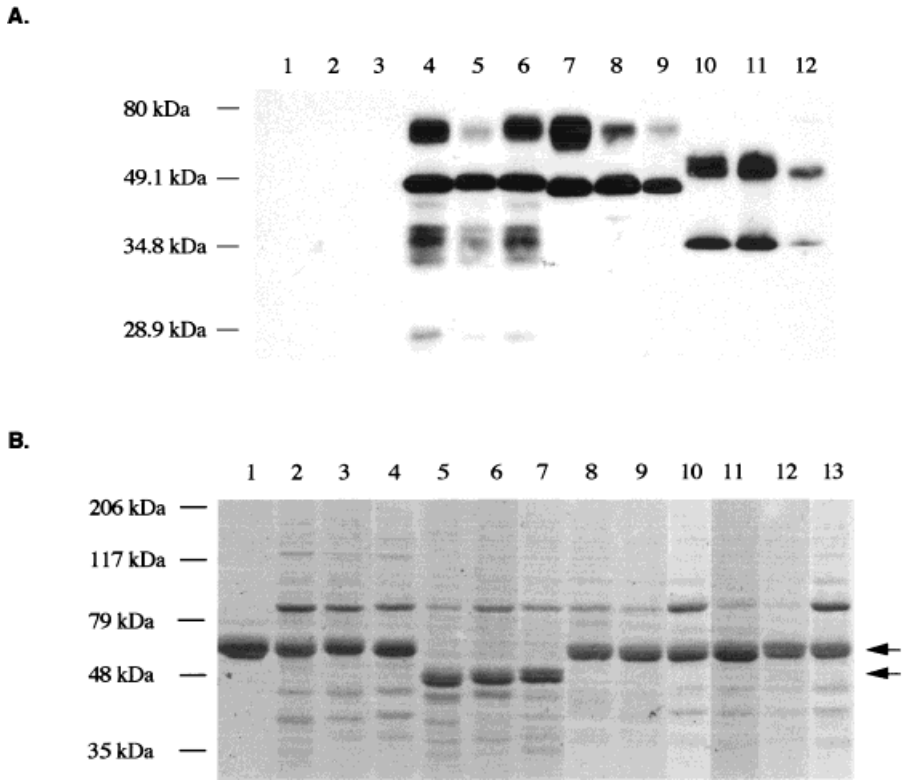

Fig. 2. Analysis of transfectants expressing STR-3_{wt}, STR-3_{cat-} or 35 kDa STR-3_{sec}. **A:** Analyses of STR-3 secretion by vector-only (**lanes 1–3**), STR-3_{wt} (**lanes 4–6**), STR-3_{cat-} (**lanes 7–9**), and 35 kDa STR-3_{sec} (**lanes 10–12**) transfectants. Aliquots of serum-free conditioned media from the individual transfectants were analyzed for STR-3 secretion by western blot. **B:** Hydrolysis of α1-PI by STR-3_{wt}, STR-3_{cat-}, and 35 kDa STR-3_{sec}. α1-PI incubated in the absence of conditioned media (lane 1) or in the presence of serum-free conditioned media from vector-only transfectants (lanes 2–4), STR-3_{wt} transfectants (lanes 5–7), STR-3_{cat-} transfectants (lanes 8–10) and 35 kDa STR-3_{sec} transfectants (lanes 11–13) is shown. The upper and lower arrows identify intact and STR-3 cleaved αl-PI, respectively.

$P = 0.002$; STR-3_{wt} vs. 35 kDa STR-3_{sec}, $P = 0.0008$, log-rank test). Taken together, these data indicate that STR-3_{wt} promotes tumor take whereas the inactive enzyme and the processed 35 kDa protein lack this activity. In addition, in the early period of observation (Days 10–20), there was a trend toward less rapid tumor development in animals injected with 35 kDa STR-3_{sec}, rather than vector-only or STR-3_{cat-}, transfectants (for example, Day 13 tumor incidence 35 kDa STR-3_{sec}, 0% [95% CI 0–0%] vs. vector-only 20% [95% CI 6–34%] and STR-3_{cat-}, 17% [95% CI 3–30%] Fig. 3).

STR-3_{wt} Tumor Cell Transfectants Have a Decreased Rate of Apoptosis In Vivo

To determine whether wild type STR-3 enhances tumor take via an effect on apoptosis, tumors from each cohort of animals were harvested on Day 24, the timepoint at which the differences in tumor incidence were most striking (Fig. 3). Representative tissue sections from STR-3_{wt}, STR-3_{cat-}, 35 kDa STR-3_{sec} and vector-only tumors were examined for apoptotic bodies and DNA fragmentation using TUNEL assays. As indicated in Table I, tumors derived from STR-3_{wt} transfectants had a significantly

TABLE I. STR-3 Tumor Cell Transfectants Have a Decreased Rate of Apoptosis In Vivo

Groups[a]	Percentage of aploptotic cells[b]
Vector-only	2.60±0.29
STR-3_{wt}	1.57±0.19[c]
STR-3_{cat-}	3.20±0.39
35 kDa STR-3_{sec}	3.01±0.57

[a]Day 24 tumors from STR-3_{wt}, STR-3_{cat-}, 35 kDa STR-3_{sec} and vector-only transfectants were harvested and representative tumor sections examined for apoptotic bodies and DNA fragmentation using TUNEL assays.
[b]The percentages of apoptotic cells in tumors derived from vector-only, STR-3_{wt}, STR-3_{cat-}, 35 kDa STR-3_{sec} are shown and compared using the paired student's t-test.
[c]STR-3_{wt} vs. Vector-only, $P < 0.01$; STR-3_{wt} vs. STR-3_{cat-}, $P < 0.05$; TR-3_{wt} vs. 35 kDa STR-3_{sec}, $P < 0.03$.

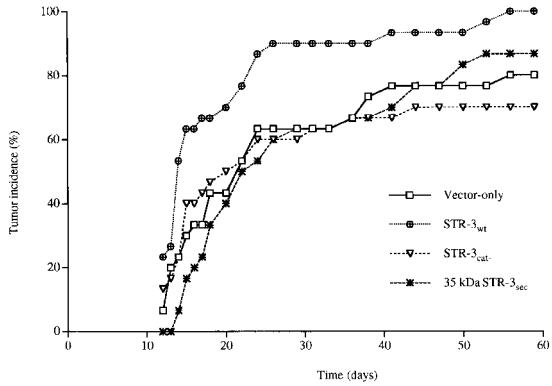

Fig. 3. Tumor incidence in animals injected with STR-3_{wt}, STR-3_{cat-} 35 kDa STR-3_{sec} or vector-only transfectants. Three independently-derived subclones of each type transfectant (STR-3_{wt}, STR-3_{cat-}, 35 kDa STR-3_{sec}, vector-only) were injected into the flanks of nude mice. Thirty animals were injected with each transfectant; 10 animals each received one of the three independently-derived subclones. Following injection, the animals were closely monitored for tumor formation. Mice were determined to have tumors on the first day that a solid mass of $\geq$25 mm^3 was identified. The tumor incidences in mice injected with STR-3_{wt}, STR-3_{cat-}, 35 kDa STR-3_{sec} and vector-only transfectants were compared using the log-rank test (STR-3_{wt} vs. STR-3_{cat-} $P = 0.002$, STR-3_{wt} vs. 35 kDa STR-3_{sec} $P = 0.0008$, STR-3_{wt} vs. vector-only $P = 0.004$).

lower percentage of apoptotic cells than tumors derived from vector-only, STR-3_{cat-} or 35 kDa STR-3_{sec} transfectants (STR-3_{wt} 1.57$\pm$0.19% vs. vector-only 2.60$\pm$0.29% [$P < 0.01$] or STR-3_{cat-} 3.20$\pm$0.39% [$P < 0.05$] or 35 kDa STR-3_{sec} 3.01$\pm$0.57 [$P < 0.03$]). Therefore, in this model system, the active STR-3 enzyme may increase tumor take by suppressing apoptosis.

DISCUSSION

We have generated a series of informative MCF-7 transfectants which express wild-type or catalytically-inactive 45 kDa STR-3 or 35 kDa processed STR-3 and analyzed their implantation and survival in nude mice. Tumors developed significantly more rapidly in animals who received STR-3_{wt}, rather than STR-3_{cat-} or vector-only transfectants, consistent with recent reports [Noel et al., 2000]. In the current studies, STR-3_{wt} transfectant tumors had a significantly low percentage of apoptotic cells, suggesting that the enzyme may promote tumor take by suppressing apoptosis.

The mechanism by which STR-3 suppresses tumor cell apoptosis remains undefined. Unlike many other MMP family members, STR-3 does not promote local invasion [Noel et al., 1996; Hotary et al., 2000] or degrade classic extracellular matrix components [Pei et al., 1994]. Other MMPs, such as MMP-1, degrade ECM collagen and expose cryptic collagen domains that facilitate the integrin-mediated binding and protection from apoptosis of tumor cells [Montgomery et al., 1994; Petitclerc et al., 1999]. Because STR-3 does not degrade classic ECM components, the enzyme may suppress tumor cell apoptosis via a different mechanism. Recent murine studies suggest that STR-3 releases an ECM-associated factor that promotes tumor take [Masson et al., 1998; Noel et al., 2000]. Our observations directly linking STR-3_{wt} overexpression with reduced tumor cell apoptosis would be consistent with this hypothesis [Masson et al., 1998; Noel et al., 2000].

However, STR-3 may have pleiotropic effects that differ in normal and malignant and in adult and embryonal cells. For example, in an amphibian model of intestinal metamorphosis, STR-3 promotes both the apoptosis of larval epithelial cells and their subsequent replacement with an adult epithelial cell layer [Ishizuya-Oka et al., 2000].

Another objective of the current study was to elucidate the role of 35 kDa STR-3 in local tumor development [Mari et al., 1998]. In contrast to the active 45 kDa enzyme, 35 kDa STR-3 does not enhance local tumor development or suppress tumor cell apoptosis. These observations are consistent with the notion that the processing of 45 kDa STR-3 to the 35 kDa protein limits STR-3 activity at the tumor/stromal interface. Because the STR-3 proform is cleaved by furin and secreted as an active 45 kDa enzyme [Pei and Weiss, 1995], 45 kDa to 35 kDa STR-3 processing likely represents a novel additional mechanism for regulating STR-3 bioactivity.

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