Stromelysin-3 Expression Promotes Tumor Take in Nude Mice

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Abstract

Stromelysin-3 (ST3) is a matrix metalloproteinase expressed in human carcinomas in ways suggesting that it may play a role in tumor progression. To test this possibility, we have performed gene transfer experiments using both anti-sense and sense ST3 expression vectors, and malignant cells either expressing (NIH 3T3 fibroblasts) or not (MCF7 epithelial cells) endogenous ST3. We have compared the ability of parental and transfected cells to cause subcutaneous tumor development in nude mice. 3T3 cells expressing anti-sense ST3 RNA showed reduced tumorigenicity, and MCF7 cells expressing mouse or human ST3 were associated with reduced tumor-free period leading to a significant increased tumor incidence ($P < 10^{-4}$). However, once established, the ST3-expressing tumors did not grow faster than those obtained with the parental MCF7 cell line. In addition, tumors obtained after sub-cutaneous injection of ST3-expressing or nonexpressing cells did not exhibit obvious histological differences, and careful examination did not reveal any local invasive tissue areas or systemic metastases. These in vivo observations were in agreement with those obtained in vitro showing that ST3 expression did not modify proliferative nor invasive properties of transfected cells. Altogether, these results indicate that ST3 expression promotes tumor take in nude mice, presumably by favoring cancer cell survival in a tissue environment initially not permissive for tumor growth. These findings represent the first experimental evidence showing that ST3 can modulate cancer progression. (J. Clin. Invest. 1996, 97:1924–1930.) Key words: cancer cell invasion/survival • metalloproteinases • tumorigenicity

Introduction

Matrix metalloproteinases (MMPs) are members of a family of zinc-dependent endopeptidases with a broad spectrum of proteolytic activities toward extracellular matrix (ECM) components (1–3). These enzymes include collagenases, gelat

1. Abbreviations used in this paper: AS, anti-sense; ECM, extracellular matrix; h, human; m ST3, human stromelysin-3; MMP, matrix metalloproteinase; S, sense; S.C., subcutaneous; TIMP, tissue inhibitor of metalloproteinase.
may have a unique role in tissue remodeling processes, including those associated with tumor progression.

In the present study, we have subcutaneously (S.C.) injected nude mice with malignant cells stably transfected with either AS or S ST3 cDNA constructs, in order to evaluate the effect of ST3 expression on tumorigenicity in vivo. Using the same cells, we have also tested whether ST3 could modulate cell proliferation and invasion in vitro.

Methods

Construction of anti-sense and sense ST3 expression vectors. The pCMV-ASmST3 plasmid was obtained by inserting in the AS orientation, a 360-bp BamHI fragment (nucleotides 1 to 360) of mouse ST3 (mST3) cDNA (24) into the pCMV vector in which expression is under the control of the cytomegalovirus (CMV) promoter (provided by B. Vogelstein, Baltimore, MD) (30). To generate the pCMVSmST3 plasmid, a 1504-bp NsiI fragment, containing the entire coding sequence of mST3, was excised and ligated to a 8-mer BamHI–NsiI adaptor and subcloned in the S orientation into the BamHI site of the pCMV vector. To generate the pCMVhST3 plasmid, a 1680-bp SphI–XhoI fragment of hST3 cDNA (17), containing the entire coding sequence, was inserted in S orientation into the compatible NheI/XhoI sites of the pCMV vector modified by insertion of a polylinker containing BamHI, NheI, and XhoI restriction sites. To generate the pSG5hST3 plasmid, a 1681 bp EcoRI fragment of hST3 cDNA was inserted in S orientation into the EcoRI site of the pSG5 vector in which expression is under the control of the SV40 promoter (31).

Obtention of stably transfected cell lines. Malignant NIH 3T3 (obtained from B. Pettmann, Strasbourg, France) (32) and MCF7 (ATCC HTB 22) cells were cultured in Dulbecco’s modified Eagle’s medium (DME) containing 10% fetal calf serum. 3T3 cells were electroporated with linearized pCMVASMmST3 construct (10 µg) or linearized pCMV vector alone (10 µg), using a BioRad apparatus at 400 volts and 125 µF. Similar conditions were used for MCF7 cell transfection with linearized pCMVSmST3 or pCMVhST3 constructs, or linearized pCMV vector alone. Linearized pSG5hST3 construct (8 µg) was co-transfected into MCF7 cells with linearized pSV2neo vector (ATCC 37149) (2 µg). Transfectants were then selected with the neomycin analogous G418 (400 µg/ml; GibCO BRL, Gaithersburg, MD) through the neomycin-resistant gene of the pCMV vector, or through the cotransfected pSV2neo vector.

RNA isolation and analysis. Total RNA was prepared from cultured cells using acid guanidinium thiocyanate-phenol-chloroform extraction (33). RNAs were fractionated by electrophoresis through 1% agarose gel in the presence of formaldehyde, and transferred to nylon membranes (Hybond N; Amersham). Filters were hybridized under stringent conditions (50% formamide, 42°C) with linearized mST3 probes 32P-labeled by random priming. Washings were performed in 2 × SSC, 0.1% SDS at 22°C, followed by 0.1 × SSC, 0.1% SDS at 55°C.

Protein analysis. Conditioned media were obtained by incubating subconfluent cells in serum-free DME. After 48 h incubation, media were collected, cleared by centrifugation at 5000 g for 30 min and precipitated at 4°C with 80% ammonium sulfate. After centrifugation at 10000 g for 2 h, protein pellets were resuspended in 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM CaCl2, 1 µM ZnCl2, and dialyzed against this buffer at 4°C for 3 h. Protein concentrations were determined using the BioRad kit (BioRad Laboratories). Protein samples were analyzed by SDS-PAGE (12%) under reducing conditions. For immunoblot analysis, proteins were transferred after electrophoresis to nitrocellulose filters which were incubated with monoclonal antibody 5T4-4C10 which recognizes the ST3 catalytic domain (35). Bound antibodies were visualized using a peroxidase-labeled goat anti-tobody raised against mouse IgG, followed by Enhanced Chemiluminescence detection (ECL kit; Dupont NEN, Boston, MA).

Tumorigenicity assay. Subconfluent NIH 3T3 cells were trypsinized, centrifugated at 1000 g for 5 min, and resuspended in serum-free DME. A total volume of 0.2 ml containing 5 × 103 cells was S.C. injected into 6–8-week-old female nude mice (Harlan, France). Subconfluent MCF7 cells were trypsinized, washed twice, and harvested by centrifugation at 1000 g for 5 min. Cells resuspended in cold serum-free DME were mixed with an equal volume of cold matrigel (10 mg/ml) prepared from the Engelbreth-Holm-Swarm tumor, as previously described (36). A total volume of 0.5 ml containing 5 × 104 cells was S.C. injected into 6–8-week-old female nude mice, previously implanted with Silastic capsules (Dow Corning) containing estradiol (37).

Injected mice were examined every 2 d for tumor apparition and tumor volume was calculated as previously described (37). Tumor incidence was defined as the percentage of mice presenting a tumor, in considering only tumors having a volume of at least 100 mm3. The data were statistically analyzed with the log Rank test (38). P values lower than 0.05 were considered as significant. All mice were autopsied. One half of each tumor was immediately frozen in liquid nitrogen for RNA analysis. The second tumor half, the lungs and the liver were fixed in phosphate buffered formalin (4%) and embedded in paraffin. Histological examination was performed on hematoxilin-eosin stained sections. Immunohistochemical localization of mST3 was performed using polyclonal antibody 812, obtained by rabbit immunization with recombinant mST3 extracted from bacterial inclusion bodies (28), whereas the immunohistochemical localization of hST3 was performed using rabbit polyclonal antibody 349 as previously described (21). Tissue sections were also stained with a rabbit polyclonal antibody raised against human pS2 (39), in order to check for the presence of metastatic MCF7 cells in mouse lungs and liver.

Cell proliferation and in vitro invasion assays. Cell proliferation was evaluated by counting trypsinized cells every 2 d using a Coulter counter (Coultronics France).

Chemoimmunassays (40) were performed using Transwell chambers (Costar Corp., Cambridge, MA) with polycarbonate membrane filters (6.5 mm diameter, 8 µm pore size) coated with matrigel (50 µg/filter) (35). Cells (3 × 104) were harvested by trypsinization, resuspended in serum-free DME containing 0.2% bovine serum albumin (ICN, Flow) and added to the upper compartment of Transwell chambers. The lower compartment was filled with 600 µl DME containing 20% fetal calf serum and 2% bovine serum albumin as chemoattractants. After 48 or 72 h of incubation at 37°C, filters were rinsed in phosphate buffered saline, fixed in paraformaldehyde 4% and permeabilized in ice-cold methanol before staining with hematoxylin-eosin. Cells on the upper face of the filters were wiped away with a cotton swab, and those on the lower face were counted on 24 fields/filter at a magnification of ×400. Three independent experiments were conducted, each using three Transwell chambers.

For collagen invasion assays, cells were seeded onto a polymerized type I collagen gel. After 24 h incubation at 37°C, the number of cells that had invaded into the collagen gel was counted as previously described (41).

Invasion assays into chick heart were based on the in vitro confrontation between cell aggregates and preculutered chick heart fragments in organ culture (42). The interaction between tested cells and chick heart fragments was evaluated by microscopic examination of serial sections fixed in Bouin Holland’s solution, embedded in paraffin, and stained with hematoxylin and eosin.

Results

Decreased tumor incidence using cells down-regulated for mST3 expression. A malignant mouse NIH 3T3 fibroblastic cell line, which constitutively expresses mST3 RNA (Fig. 1, lane 1), was stably transfected with the pCMVASmST3 vector (see Meth-
Figure 1. Northern blot analysis of NIH 3T3 cells transfected with an AS mST3 cDNA construct. (Lane 1) parental cells; (lanes 2 and 3) pCMV transfected cells; (lanes 4–8) pCMVASmST3 transfected cells. The position of the endogenous sense (S) and exogenous anti-sense (AS) mST3 transcripts are indicated. The 36B4 probe was used as a control for loading and transfer; autoradiography was for 2 d (mST3) and 18 h (36B4).

Table I. Tumorigenicity of Parental, pCMV and pCMVASmST3-transfected NIH 3T3 Cells, after S.C. Injection into Nude Mice

<table>
<thead>
<tr>
<th>Clones</th>
<th>Transfected vector</th>
<th>S RNA*</th>
<th>AS RNA*</th>
<th>3 wk</th>
<th>6 wk</th>
<th>8 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3</td>
<td></td>
<td>+</td>
<td>−</td>
<td>5/10 (50%)</td>
<td>8/10 (80%)</td>
<td>9/10 (90%)</td>
</tr>
<tr>
<td>3T3/16.1</td>
<td>pCMV</td>
<td>+</td>
<td>−</td>
<td>1/5 (20%)</td>
<td>5/5 (100%)</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>3T3/14.3</td>
<td>pCMV</td>
<td>+/−</td>
<td>−</td>
<td>0/7 (0%)</td>
<td>0/7 (0%)</td>
<td>1/7 (14%)</td>
</tr>
<tr>
<td>3T3/7.5</td>
<td>pCMVASmST3</td>
<td>+</td>
<td>+</td>
<td>0/10 (0%)</td>
<td>2/10 (20%)</td>
<td>3/10 (30%)</td>
</tr>
<tr>
<td>3T3/4.6</td>
<td>pCMVASmST3</td>
<td>+</td>
<td>+</td>
<td>0/7 (0%)</td>
<td>1/7 (14%)</td>
<td>4/7 (57%)</td>
</tr>
<tr>
<td>3T3/3.6</td>
<td>pCMVASmST3</td>
<td>+</td>
<td>+/−</td>
<td>3/7 (43%)</td>
<td>7/7 (100%)</td>
<td>7/7 (100%)</td>
</tr>
<tr>
<td>3T3/2.1</td>
<td>pCMVASmST3</td>
<td>+</td>
<td>−</td>
<td>6/12 (50%)</td>
<td>9/12 (80%)</td>
<td>11/12 (92%)</td>
</tr>
<tr>
<td>3T3/1.1</td>
<td>pCMVASmST3</td>
<td>+</td>
<td>−</td>
<td>1/7 (14%)</td>
<td>3/7 (43%)</td>
<td>5/7 (71%)</td>
</tr>
</tbody>
</table>

* and AS mST3 RNA expression was evaluated by Northern blot analysis as illustrated in Fig. 1; (+), high, (+/−), low and (−), undetectable levels.

Each ratio in the table represents the number of tumors larger than 100 mm³ vs. the total number of injected mice, at the indicated time after cell injection; corresponding percentages are indicated in parentheses.
moderate (MCF7mST3/8; Fig. 2A, lane 4) levels of mST3 RNA, whereas we could not detect any mST3 RNA in the MCF7mST3/5 clone (Fig 2 A, lane 5), even after a longer exposure time (data not shown). By Western blot analysis, we could detect mST3 protein in culture media conditioned by MCF7mST3/1, MCF7mST3/11 and MCF7mST3/8 cells (Fig. 2 B, lanes 2–4), but not by MCF7mST3/5 cells (Fig. 2 B, lane 5). Both pro- and mature mST3 forms were detected together with some degradation products, including the 28-kD form.

In vivo tumorigenicity of these cells was tested by S.C. injection in nude mice. As shown in Fig. 2 C, the tumor incidence in mice injected with MCF7 cells expressing mST3 was significantly increased (log Rank test, \( P < 2 \times 10^{-5} \)). Thus, 20 d after cell injection, MCF7mST3/1, MCF7mST3/11 and MCF7mST3/8 cells gave rise to a tumor incidence of 50–62%, whereas parental MCF7 cells and MCF7mST3/5 cells led to a tumor incidence of 25 and 12%, respectively. This difference in tumor incidence, which was still observed 35 d after cell injection, was no longer apparent after 40 d when all cells, excepting MCF7mST3/5, led to 100% tumors. Thus, the variation in tumor incidence which was observed in these experiments in fact corresponds to a reduction of the tumor-free period when mST3-expressing cells were S.C. injected in mice.

These findings were in good agreement with our hypothesis that mST3 could promote tumorigenicity in nude mice. However, since differences have been observed between the functional properties of mST3 and hST3 (28, 43), we tested whether similar results could be obtained using MCF7 cells expressing hST3.

Increased tumor incidence using cells over-expressing hST3. Using the same protocol as described above, we obtained 3 MCF7 clones stably transfected with the pCMVhST3 vector and expressing hST3 RNA (MCF7hST3/3, MCF7hST3/8 and MCF7hST3/9; Fig. 3 A, lanes 4–6). These cells secreted both pro- and mature hST3 in their culture media (Fig. 3 B, lanes 4–6). In addition, we obtained two control clones (MCF7pCMV/1 and MCF7pCMV/2) transfected with the pCMV vector alone, which did not express hST3 (Fig. 3 A, lanes 2 and 3). As observed for mST3, hST3 expression was found to promote tumor incidence in nude mice (log Rank test, \( P < 4 \times 10^{-4} \)) (Fig. 3 C). Similar results were also obtained using a clone (MCF7hST3/13) stably transfected with a construct in which hST3 was expressed under the control of the less potent SV40
promoter of the pSG5 vector, instead of the CMV promoter. Low levels of hST3 RNA (Fig. 4A, lane 2) and of the hST3 28kD form (Fig. 4B, lane 2) could be detected in MCF7hST3/13 cells and culture media, respectively. Despite this low level of hST3 expression, MCF7hST3/13 cells led to an increased tumor incidence (log Rank test, \( P < 4 \times 10^{-21} \)). Thus, 20 d after cell injection, 75% of mice injected with MCF7hST3/13 cells exhibited tumors whereas parental MCF7 cells gave rise to only 25% (Fig. 4C).

**ST3 over-expression did not modify cell growth and invasiveness.** To define what could be the process leading to a reduction of the tumor-free period and thus to an increased tumor incidence after S.C. injection of MCF7 cells expressing either mST3 or hST3, we first evaluated tumor growth and cell proliferation rates. We observed that once the tumors were established, the evolution of tumor volumes was identical from one cell clone to another, independently of the levels of ST3 expression. Consistently, no major differences were observed for the in vitro cell proliferation rates whether or not the transfected cells expressed mST3 or hST3 (data not shown).

We next tested the invasive properties of transfected MCF7 cells, both in vivo and in vitro. All clones grew as well delineated tumor masses, and no evidence of local invasion could be detected regardless of the tumor size. Moreover, careful anatomopathological examination of mice did not reveal any lung or liver metastases, even after pS2 immunostaining allowing the detection of isolated MCF7 cells (39). No histological differences, notably concerning their vascular status, were observed between tumors obtained in nude mice after injections of either parental MCF7 cells or cells transfected with pCMV, pCMVmST3 or pCMVhST3. Although the tumor stroma was not as well developed as that of human breast carcinomas, we could observe areas where tumoral epithelial cells were surrounded by fibroblastic cells after injection of ST3-expressing (Fig. 5A) or control MCF7 cells (Fig. 5B). Endogenous mST3 could not be detected in these fibroblasts, using antibody 612 directed against mST3 (data not shown). MCF7 cells expressing recombinant ST3 protein were found to be homogeneously distributed throughout tumors, and staining was restricted to the cytoplasm of transfected MCF7 cells (Fig. 5C).
In the present study, we investigated the role of ST3 in the development of tumors after S.C. injection of malignant cells in nude mice. We demonstrated that the incidence of tumors obtained using malignant mouse fibroblastic NIH 3T3 cells, which endogenously express ST3, was decreased when high levels of AS mST3 RNA were expressed in these cells. Inversely, we observed that human epithelial MCF7 breast cancer cells, which do not express endogenous ST3, showed significant increased tumorigenicity in nude mice when stably transfected in order to produce mST3 (P < 2 × 10⁻⁵). Similar observation was made using MCF7 cells stably transfected in order to express hST3 at either high levels under the control of the CMV promoter (P < 4 × 10⁻⁴) or at lower levels under the control of the SV40 promoter (P < 4 × 10⁻³). These studies showed that the increased tumor incidences induced by either mST3 or hST3 resulted from a shortening of tumor-free periods indicating that, in nude mice, ST3 is involved in tumor formation rather than in tumor growth. How could ST3 modulate tumor development in nude mice? In vitro analyses of cell proliferation did not show any obvious differences between ST3-expressing and non-expressing cells, suggesting that the observed increase in tumor incidence could not be ascribed to an increased growth rate of ST3-expressing cells. Consistently, once the tumors were established in nude mice, their proliferation rates appeared to be independent of their levels of ST3 expression. In addition, ST3 does not seem to be implicated in cell invasion, since MCF7 cells expressing ST3 did not exhibit any modifications of their capacity to invade interstitial-like or basement membrane matrices. Accordingly, no local invasion or metastatic spreading was detected by histological analysis of tumors, lungs and livers of nude mice injected with ST3-expressing MCF7 cells. Taken together, these findings indicate that ST3 expression did not favor tumor incidence in nude mice by increasing cell proliferation or invasion, but rather by increasing tumor take. This is consistent with our finding that the increase in tumor incidence observed for ST3-expressing MCF7 cells was more pronounced in the first weeks after cell injection. Thus, we propose that ST3 contributes to cell survival and implantation in host tissues. This hypothesis may appear paradoxical with regards to previous observations showing that ST3 was expressed during processes involving epithelial cell apoptosis such as mouse mammary gland involution (25), mouse limb, tail and snout morphogenesis (23) and frog metamorphosis (24). However, while unwanted cells are eliminated during apoptotic events, some neighboring cells are selected for survival (44 and references therein). In this respect, we note that ST3 gene expression is also observed at the time of epithelial cell proliferation occurring in the neoformation of mammary ducts following mammary gland involution (25), in developing limb, tail and snout until birth (23), and in proliferative endometrium (26). Interestingly, comparable observations have been reported for matrilysin, another MMP implicated in cancer progression (1, 3). Thus, while SW480 colon cancer cells stably transfected to express matrilysin did not show in vitro any modification of their proliferative or invasive properties, these cells exhibited increased tumorigenicity in vivo, suggesting that the most significant role of matrilysin was at early stage of tumor progression (14).

Tumors that are obtained by S.C. injection of malignant cells into nude mice should be regarded as a model for metastatic implantation rather than for primary tumor development (45). Indeed, S.C. injected cells mimic metastatic cells after extravasation, when these cells have to successfully implant at the metastatic site before being able to proliferate and invade (45, 46). This process is dependent upon the cells’ capability to survive in host tissues, either in an independent manner or by the recruitment of required factors from their near vicinity. In this context, ST3 may represent a local factor contributing to the survival and implantation of ST3-expressing MCF7 cells S.C. injected in nude mice. It appears reasonable to believe that this might be also the case in human tumors, where ST3 is produced by tumor stromal cells and not by the cancer cells themselves (17–22). ST3 is a secreted protein which is specifically expressed by fibroblastic cells located in the vicinity of cancer cells, suggesting that the same effects should be observed irrespective of the source of the protein. Thus, in human carcinomas ST3 could act as a paracrine host factor contributing to cancer cell survival outside of their compartment of origin. Consistently, in normal embryonic and adult tissues ST3 expression has been reported to occur during tissue remodeling processes, notably when, as in malignant processes, the integrity of the basement membrane which separates epithelial cell compartments from mesenchymal cells showed failure, leading to epithelial/stromal cell contacts (20, 23, 25, 26). Although further studies are required to evaluate this possibility, a role for ST3 in favoring cell survival is consistent with observations demonstrating that high ST3 expression levels were associated with metastatic propensity in human carcinomas (22, 47).

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