Long-term exposure to elevated levels of circulating TIMP-1 but not mammary TIMP-1 suppresses growth of mammary carcinomas in transgenic mice

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Tissue inhibitor of metalloproteinases-1 (TIMP-1) regulates matrix metalloproteinase activity, acts as a growth stimulator and inhibits apoptosis. We developed transgenic mice to evaluate the relevance of circulating versus mammary TIMP-1 in mammary carcinogenesis. The transgene was placed under the control of the albumin (Alb) promoter for the production of large amounts of TIMP-1 in the liver and release into the systemic circulation to achieve chronically elevated blood levels. The initial 7,12-dimethylbenz[a]anthracene (DMBA) mammary carcinogenesis study showed greatly decreased tumor incidence in heterozygous Alb-TIMP-1 mice (25%), compared with their wild-type (wt) littermates (83.3%). Metastatic mammary carcinomas were induced in the Alb-TIMP-1 mice through breeding with mice expressing the polyomavirus Middle T antigen (MT) under the control of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR). Both the mammary tumor burden and the incidence of lung metastases were lower in the Alb-TIMP-1/MMTV-MT mice than their MMTV-MT littermates. Analysis of the Alb-TIMP-1/MMTV-MT tumors showed evidence of decreased proliferative activity and inhibition of apoptosis, whereas microvascular density was not affected. Transgenic expression of TIMP-1 in mammary epithelial cells was accomplished by using MMTV-LTR. In contrast to the Alb-TIMP-1 mice, there was insignificant difference in the growth of both DMBA- and MT-induced mammary tumors between heterozygous MMTV-TIMP-1 mice and their wt littermates. The MT-induced mammary tumors of the MMTV-TIMP-1 mice were separated into ‘low’ and ‘high’ TIMP-1 expressing groups. The ‘high’ TIMP-1 expressing tumors exhibited significantly higher proliferative activity than the tumors of the MMTV-MT only mice, whereas the number of apoptotic cells and microvascular density were not different. The findings of this study show that circulating TIMP-1, but not mammary-derived TIMP-1, has growth suppressive effects on DMBA and MT-induced mammary carcinomas.

Introduction

Under physiological conditions, extracellular matrix (ECM) turnover is tightly regulated by coordinated expression of proteolytic enzymes, including matrix metalloproteinases (MMPs) and their endogenous inhibitors, TIMPs (1,2). The MMP/TIMP-1 balance is often lost in malignant tumors, resulting in increased degradation of ECM, including basement membrane (3,4). Tissue inhibitor of metalloproteinases-1 (TIMP-1) is a secreted glycoprotein, which is present in body fluids and the ECM (5). When the TIMP-1 sequence was first determined in 1986 (6), it was found to be identical to that of erythroid precursor cells (7). TIMP-1 is also a mitogen for various other cell types (8–11). Additionally, TIMP-1 has been established as an inhibitor of angiogenesis (12–14) and a survival factor (15–21). Translocation of TIMP-1 to the nucleus may reflect events involved in its growth promoting or anti-apoptotic functions (22). TIMP-1 has paradoxical effects on tumor progression in experimental models. Recombinant TIMP-1 (rTIMP-1) inhibits experimental metastasis of murine melanoma cells (23) and ras-transfected embryonic stem cells (24). Over-expression of TIMP-1 is associated with decreased tumorigenicity and metastatic capability of different tumor cell types (25–27) and down-regulation of TIMP-1 confers oncogenicity and increased invasiveness on Swiss/3T3 cells and embryonic stem cells (28,29). Conversely, over-expression of TIMP-1 in rat mammary carcinoma cells results in up-regulation of vascular endothelial growth factor (VEGF) and increased growth in nude mice (30). Also, TIMP-1 expression in tumorigenic cells can either increase or suppress lung metastases (31) and TIMP-1 expression is increased in various types of human malignancies, including breast cancer (32–35). High TIMP-1 levels are associated with poor prognosis in lymphoma (36) and carcinomas of breast (37–41), lung (42), stomach (43), colon (44), bladder (45) and prostate (46). In invasive breast cancer, the TIMP-1 signal is present in both tumor and stromal cells with the most intense signals at the tumor-stromal interface (35).

A number of synthetic MMP inhibitors have been developed, which showed antitumor activity in murine models (47–49). Regrettably, these inhibitors failed to show therapeutic benefits in clinical cancer trials (50). We have established transgenic mice to further evaluate the effects of chronically
elevated TIMP-1 levels, either in the circulation or the mammary gland, on mammary tumor growth and metastasis.

Materials and methods

Generation of transgenic mice

Full-length human TIMP-1 cDNA (624 bp, +63 to +687) was isolated from the HT-1080 fibrosarcoma cell line and cloned into the EcoRI and NotI sites of the cytomegalovirus (CMV) promoter driven pcDNA vector (Promega, Madison, WI) as described previously (30). The CMV promoter was then deleted from the vector by digestion with BglII and PstI and replaced with the mouse albumin (Alb) promoter containing the enhancer and proximal promoter regions (51) to produce the Alb-TIMP-1 vector. The mouse mammary tumor virus (MMTV)-TIMP-1 vector was made by deleting the CMV promoter from the pcDNA vector with EcoRI and SalI. It was replaced with MMTV-long terminal repeat (LTR) obtained from the pMMMneo vector (Clontech, Palo Alto, CA). Verification of the transgene constructs was done by restriction mapping and nucleotide sequencing. Linearized Alb-TIMP-1 (3.7 kb HpaI fragment) or MMTV-TIMP-1 (3.7 kb BspHI, DraIII fragment) were injected into fertilized eggs from C57BL/6 J-CBA (B6CBA) hybrid mice. Founders were identified by Southern blot analysis of digested genomic tail DNA. The DNA was separated in 0.8% agarose gel, transferred to nylon membranes, and hybridized with 32P-labeled TIMP-1 cDNA probe, consisting of a 930 bp fragment digested with EcoRI and PvuI. Heterozygous Alb-TIMP-1 and MMTV-TIMP-1 lines were developed from the founders with the Alb-TIMP-1 hRNA expression in liver and mammary glands, determined by northern blot analysis.

Transgenic offspring were identified using PCR analysis of genomic tail DNA. The PCR conditions were: 30 cycles at 94°C for 1 min, 50°C for 2 min and 72°C for 3 min. The primers for both Alb-TIMP-1 and MMTV-TIMP-1 were: GAGACTTTAATACGACTCACTATAGG (sense) and GCGGCCG-CCGATTCCGACCTACATAGG (sense) and GCGGCCG-CCGATTCCGACCTACATAGG (antisense). The mice were maintained and then killed according to NIH guidelines for animal care. Tissues were prepared for immunohistochemistry for mammary tumor detection for 30 weeks. At the end of the study, the mice were killed and necropsied. The major organs were subjected to macroscopic and microscopic examination. A second DMBA study was undertaken in haploid Alb-TIMP-1 and 14 wt littermates. These mice received a single dose of MPA (100 mg) in a slow release pellet at 6 weeks of age. The same DMBA dosing regimen was used as in the Alb-TIMP-1 mice. The mice were killed 10 weeks after the last MDA dose. All of the mammary tumors were excised and weighed.

For induction of metastatic mammary carcinomas, heterozygous Alb-TIMP-1 mice and 12 wild-type (wt) littermates, initially received subcutaneous implants of two 25 mg, 90-day release pellets of medroxyprogesterone (MPA) (Innovative Research of America, Sarasota, FL). Administration of DMBA (50 mg/kg, dissolved in corn oil) by gavage was started at 9 weeks of age and continued weekly for 5 consecutive weeks. The mice were monitored by biweekly examination for mammary tumor formation from 5 weeks. At the end of the study, the mice were killed and necropsied. The major organs were subjected to macroscopic and microscopic examination. A second DMBA study was undertaken in 16 heterozygous MMTV-TIMP-1 and 14 wt littermates. These mice received a single dose of MPA (100 mg) in a slow release pellet at 6 weeks of age. The same DMBA dosing regimen was used as in the Alb-TIMP-1 mice. The mice were killed 10 weeks after the last MDA dose. All of the mammary tumors were excised and weighed. For induction of metastatic mammary carcinomas, heterozygous Alb-TIMP-1 and MMTV-TIMP-1 females were crossed with MMTV-Midd T antigen (MT) males (52), which were kindly provided by Dr William J.Muller (McMaster University). Genotyping for MT was performed by PCR of genomic tail DNA. The PCR primers and conditions for the MT construct have been described elsewhere (52). All animals were killed at 18 weeks of age and necropsied. All mammary tumors were excised and weighed. Mann–Whitney U-test was used to calculate the median values of tumor weights of the DMBA- and MT-induced mammary carcinomas in the Alb-TIMP-1 and MMTV-TIMP-1 mice.

The two largest tumors from each mouse were cut into pieces and either snap frozen and stored at −80°C, or fixed in 10% buffered formalin for H&E or special staining. The lungs were inflated with 10% buffered formalin. Four H&E stained cross sections of the lungs, cut 50 μm apart, were used to enumerate micro- and macrometastases. The average surface area of individual metastases was also determined, using images taken at 7.5× magnification, and stored by Nikon ACT-1 software. The metastases were captured with Adobe Photoshop software, counted, and measured, using NIH Image 1.62f software.
of CD-31, using anti-mouse CD31 monoclonal antibody (BD PharMingen, San Diego, CA) and the ABC method (Vector Laboratories, Burlingame, CA). Proliferative and apoptotic indices were determined by counting positive cells in 15 random microscopic fields at 400× magnification. The microvascular density was assessed by counting CD-31 positive microvessels in 15 fields at 200× magnification, choosing the areas with the highest vascular density. Student’s t-test was used for statistical analyses of cell proliferation, apoptosis and microvascular density.

Results

Mouse models and biological activity of transgenic TIMP-1

The Alb enhancer/promoter was used to target the liver for production of large amounts of transgenic TIMP-1 and chronic release into the systemic circulation (Figure 1A). Of the five Alb-TIMP-1 founders produced, the founder with the highest hepatic hTIMP-1 expression was chosen for the development of a transgenic line. Northern blot analysis revealed that transgenic TIMP-1 expression was restricted to the liver (Figure 1B). The highest human TIMP-1 plasma levels, ~800 ng/ml, were observed at 2 weeks of age. The levels then dropped sharply to ~500 ng/ml at 4 weeks of age (Figure 1C), which reflects a decrease in the activity of the Alb promoter during the first 6 weeks after birth (51). The TIMP-1 plasma levels were then maintained at 400–500 ng/ml for the remainder of the 30-week experimental period.

Reverse zymography was used to assess total TIMP-1 MMP inhibitory activity in plasma samples. Consistent with the presence of transgenic TIMP-1, in addition to mouse TIMP-1, in the plasma, the Alb-TIMP-1 mice possessed 2–3-fold higher circulating TIMP-1 activity than the wt littermates at 6 weeks of age (Figure 1D), suggesting that transgenic TIMP-1 contributed to the MMP-9 inhibitory activity. Suggestive of continuous release of transgenic TIMP-1 from the liver into the circulation, a positive relationship was found between the liver and plasma levels of TIMP-1 in a group of nine 6-week-old Alb-TIMP-1 mice (Figure 1E). No macro- or microscopic abnormalities were detected in major organs of the Alb-TIMP-1 mice (examined up to 6 months of age) (not shown). Furthermore, results of liver function tests in all age groups of the heterogenous Alb-TIMP-1 mice were within normal limits (data not shown).

For targeting of the mammary gland, human TIMP-1 cDNA was placed under the control of the MMTV-LTR (Figure 2A). Consistent with the reported lack of organ specificity of the MMTV promoter in transgenic models (54), TIMP-1 expression was observed in salivary glands, lungs and spleen, besides mammary glands in the MMTV-TIMP-1 mice (Figure 2B). An age-dependent increase in TIMP-1 levels was observed in mammary glands of heterozygous MMTV-TIMP-1 mice (Figure 2C). This is consistent with tissue re-organization, associated with ductal branching during mammary gland development. Similarly, a strong MMP-9 inhibitory zone at a molecular weight of ~29 kDa, corresponding to TIMP-1, was observed in mammary glands of 6-week-old MMTV-TIMP-1 mice.
mice, whereas TIMP-1 activity was not detected in mammary glands of the wt littermates (Figure 2D). Microscopic examination of whole mount preparations of mammary glands from 6 to 20-week-old virgin MMTV-TIMP-1 female mice and the wt littermates showed no abnormalities in ductal branching or terminal end bud development (not shown). Similarly, no histological abnormalities were observed in H&E stained sections of mammary glands from pregnant and lactating MMTV-TIMP-1 mice (not shown). Normal size and excellent general health of the pups of heterozygous Alb-TIMP-1 and MMTV-TIMP-1 mice further established that mammary gland development and lactation was not affected by the transgenic TIMP-1.

Circulating but not mammary transgenic TIMP-1 suppresses tumor growth

In order to determine the effect of circulating TIMP-1 on mammary carcinogenesis, the Alb-TIMP-1 and MMTV-TIMP-1 mice were either given DMBA orally or crossed with MMTV-MT-transgenic mice. In the initial DMBA mammary carcinogenesis study, the appearance of macroscopic tumors was recorded biweekly for a 30-week experimental period in heterozygous Alb-TIMP-1 mice and their wt littermates. During the 30-week study, macroscopic mammary carcinomas were detected in four of 16 (25%) of the Alb-TIMP-1 mice and 10 of 12 (83.3%) of the wt littermates ($P = 0.0032$) (Figure 3A). Also, the total number of tumors was lower in the Alb-TIMP-1 mice (four tumors) than the wt mice (21 tumors). However, the time until macroscopic tumors were first detected (10 weeks after the last DMBA dose) was not different in the two groups. A separate 12-week study was undertaken to determine if TIMP-1 suppressed the early stages of DMBA-induced mammary carcinogenesis in the Alb-TIMP-1 mice. Microscopic examination of H&E stained sections of mammary gland whole mounts, prepared at 4, 8 and 12 weeks post-DMBA, showed no difference in the number, size or microscopic appearance of hyperplastic lesions and early carcinomas between the Alb-TIMP-1 and wt groups (not shown). The data suggest that TIMP-1 did not interfere with the initial stages of DMBA-induced mammary carcinogenesis. They also ruled out that impairment of metabolic activation of DMBA was responsible for the decreased tumor incidence.

Alb-TIMP-1 mice were crossed with MMTV-MT transgenic mice to generate metastatic mammary carcinomas. At the end of the 18-week experimental period, tumor incidence was 100% in both Alb-TIMP-1/MMTV-MT mice and the MMTV-MT only littermates. Similarly, there was insignificant difference in the average tumor multiplicity (eight and nine per mouse) between the two groups. However, the median value of the combined tumor weight per mouse was significantly lower in the Alb-TIMP-1/MMTV-MT mice ($5.68 \pm 0.79$ g) than the MMTV-MT only mice ($9.72 \pm 1.09$ g; $P < 0.01$) (Figure 3B). Also, the median weight per tumor was significantly lower in the Alb-TIMP-1/MMTV-MT ($0.71 \pm 0.12$ g) than the MMTV-MT only mice ($1.06 \pm 0.12$ g; $P < 0.01$). Micro- and macroscopic lung metastases were detected in 17 of the 23 Alb-TIMP-1/MMTV-MT mice (73.9%) and 22 of the 23 MMTV-MT only mice (95.7%; $P = 0.042$) (Table I). However, the mean number and the average area of lung metastases per mouse and the mean area of individual metastasis were not statistically different in the two groups (Table I).

DMBA and MT-induced mammary carcinogenesis studies were also carried out in the MMTV-TIMP-1 mice. The DMBA study, which was terminated 10 weeks after the last DMBA dose, showed insignificant difference in the combined tumor weight ($P = 0.92$) between the MMTV-TIMP-1 mice and wt
littermates (Figure 3C). Also, an 18-week MT-induced mammary carcinogenesis study showed no difference in the combined tumor weight between the MMTV-TIMP-1/MMTV-MT and the MMTV-MT only littermates (Figure 3D). Furthermore, the incidence of lung metastases, the average number and the average area of metastases were not significantly different in the two groups (Table I).

The histopathology of the mammary carcinomas induced by DMBA and MT was that of adenocarcinoma with papillary and tubular growth patterns except for a few DMBA-induced carcinomas in the Alb-TIMP-1 mice, which displayed squamous differentiation (not shown). TIMP-1 had no impact on the growth patterns of the carcinomas, the amount of tumor stroma, or the extent of type IV collagen and laminin immunopositivity in the tumor stroma (not shown).

Transgenic TIMP-1 levels were compared in size-matched tumors of the Alb-TIMP-1/MMTV-MT and MMTV-TIMP-1/MMTV-MT only mice. The levels were variable within each group and a big difference was observed between the groups (Figure 4A and B). The highest TIMP-1 level in the Alb-TIMP-1/MMTV-MT group was ~6.4 ng/mg (Figure 4A). In the MMTV-TIMP-1/MMTV-MT group, the mammary
tumors were divided into ‘high’ and ‘low’ subgroups (Figure 4B). The highest TIMP-1 level in the ‘low’ subgroup of 13 tumors was ~35 ng/mg and the highest level in the ‘high’ subgroup of six tumors was close to 10 000 ng/mg. At the end of the experimental period, the average transgenic TIMP-1 plasma level in the tumor bearing MMTV-TIMP-1/MMTV-MT group was considerably higher than the average TIMP-1 level in the ALB-TIMP-1/MMTV-MT group (Figure 4C). Interestingly, tumor growth was not suppressed by the high tumor and plasma TIMP-1 levels in the MMTV-TIMP-1/MMTV-MT group. The increase in TIMP-1 activity in the tumors and plasma of the MMTV-TIMP-1/MMTV-MT mice is demonstrated by reverse zymography of representative samples of size matched tumors in Figure 4D. A faint MMP-9 inhibitory TIMP-1 activity was observed in the MMTV-MT only tumors (lanes 1 and 2), compared with the MMTV-TIMP-1/MMTV-MT tumors (lanes 3 and 4). Similarly, TIMP-1 activity was barely detected in the plasma from the tumor bearing MMTV-MT only mice (lanes 5 and 6), compared with strong TIMP-1 activity in the plasma from the MMTV-TIMP-1/MMTV-MT mice (lanes 7 and 8).

The possibility was examined that over-expression of TIMP-1 could affect MMP expression and activities in the MT-induced mammary carcinomas. RT--PCR analysis showed no difference in MMP-2 and MMP-9 mRNA levels in tumors from the transgenic and MMTV-MT only littermates (not shown). Zymographic studies of lysates from individual tumors showed variable gelatinolytic MMP-2 and MMP-9 activities and no clear difference between the TIMP-1 transgenic and MMTV-MT only groups (not shown). However, quantitative analysis of active MMP-2 and MMP-9 in individual size-matched tumors showed that the Alb-TIMP-1/MMTV-MT group displayed lower levels of active MMP-2 ($P < 0.05$)

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<tr>
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<th>No. of mice with metastases</th>
<th>Average no. of metastases</th>
<th>Average area of metastases (mm$^2$)</th>
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<tr>
<td>MMTV-MT</td>
<td>22 (95.7%)</td>
<td>47.22 ± 45.46</td>
<td>0.078 ± 0.158</td>
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<tr>
<td>Alb-TIMP-1/MMTV-MT</td>
<td>17 (73.9%)</td>
<td>25.52 ± 35.64</td>
<td>0.139 ± 0.350</td>
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<td>$P$ value</td>
<td>0.042</td>
<td>0.109</td>
<td>0.448</td>
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<tr>
<td>MMTV-MT</td>
<td>11 (91.7%)</td>
<td>39.48 ± 33.41</td>
<td>0.074 ± 0.040</td>
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<tr>
<td>MMTV-TIMP-1/MMTV-MT</td>
<td>12 (100%)</td>
<td>26.44 ± 29.14</td>
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<tr>
<td>$P$ value</td>
<td>0.133</td>
<td>0.230</td>
<td>0.440</td>
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Comparison of incidence, number and area of lung metastases between MMTV-MT ($n = 23$) and Alb-TIMP-1/MMTV-MT ($n = 23$) mice or MMTV-MT ($n = 12$) and MMTV-TIMP-1/MMTV-MT ($n = 12$) mice. Shown are the number and percentage (%) of mice with metastases, the average number of metastases ± SD and the average area (mm$^2$) of metastases ± SD. $P$ value was calculated with Mann-Whitney $U$-test.

Fig. 4. Increased hTIMP-1 and decreased active MMP-2/MMP-9 in Alb-TIMP-1/MMTV-MT tumors. (A) hTIMP-1 levels were measured by ELISA in (A) individual mammary tumors of Alb-TIMP-1/MMTV-MT ($n = 8$) and MMTV-MT only mice ($n = 8$), (B) mammary tumors of MMTV-TIMP-1/MMTV-MT and MMTV-MT only littersmates. Based on the hTIMP-1 concentration, the MMTV-TIMP-1/MMTV-MT tumors were divided into two groups, designated as ‘Low’ ($n = 13$) and ‘High’ ($n = 6$) expressing tumors. (C) Plasma hTIMP-1 levels in tumor bearing Alb-TIMP-1/MMTV-MT ($n = 22$) and MMTV-TIMP-1/MMTV-MT mice ($n = 7$). (D) Reverse zymography shows MMP-9 inhibitory TIMP-1 activity in representative tumor and plasma samples from: (1,2) MMTV-MT only tumors; (3,4) MMTV-TIMP-1/MMTV-MT tumors; (5,6) plasma from tumor bearing MMTV-MT only mice; (7,8) plasma from tumor bearing MMTV-TIMP-1/MMTV-MT mice. (E) Active MMP-2 and MMP-9 levels in size-matched transgenic Alb-TIMP-1/MMTV-MT, MMTV-TIMP-1/MMTV-MT and MMTV-MT only tumors. The data show the average levels (ng/g protein) of active MMP-2 and MMP-9 in four tumors from each group. (F) $P$ value was calculated with Mann-Whitney $U$-test.
and MMP-9 ($P = 0.18$) than the MMTV-MT only group (Figure 4E and F). However, there were insignificant differences in the levels of active MMP-2 and MMP-9 between the MMTV-TIMP-1/MMTV-MT and MMTV-MT only groups (Figure 4E and F). Zymographic analysis of caseinolytic MMP-3 in MT-induced tumors of the Alb-TIMP-1 and MMTV-TIMP-1 mice showed variable activities and minor differences from the MMTV-MT littermate groups (not shown).

The Alb-TIMP/MMTV-MT and the MMTV-TIMP/MMTV-MT tumors displayed different distribution of TIMP-1 (Figure 5). In the Alb-TIMP/MT tumors, TIMP-1 positivity was mostly deposited in the tumor stroma (Figure 5A). In contrast, TIMP-1 was more diffusely distributed in the MMTV-TIMP-1/MT tumors, which is consistent with the release of transgenic TIMP-1 by individual carcinoma cells into the surrounding environment (Figure 5B). TIMP-1 immunopositivity was not detected in MMTV-MT only tumors (Figure 5C and D).

Circulating and mammary TIMP-1 have opposite effects on tumor cell proliferation

Immunohistochemical analysis of size-matched Alb-TIMP-1/MMTV-MT and MMTV-MT only tumors was carried out to measure proliferative and apoptotic activities, as well as microvascular density. The average number of proliferating tumor cells, judged by PCNA positivity, was significantly lower in the Alb-TIMP-1/MMTV-MT mice than in the MMTV-MT only mice ($P < 0.01$) (Figure 6A). There was also a significant decrease in the average number of apoptotic tumor cells in the Alb-TIMP-1/MMTV-MT mice ($P < 0.001$) (Figure 6B). Surprisingly, insignificant difference in microvascular density was observed between the Alb-TIMP-1/MMTV-MT and MMTV-MT only tumors (Figure 6C) and semi-quantitative RT-PCR analysis of CD31 mRNA levels showed no difference between the two groups (not shown). More data on angiogenesis associated factors are described in the next paragraph on MMTV-TIMP-1 tumors.

The ‘high’ TIMP-1 expressing MMTV-MT/MMTV-MT tumor group, but not the ‘low’ expressing group, showed a significant increase in the number of PCNA positive cells, compared with the MMTV-MT only group ($P < 0.05$) (Figure 7A). An association between the transgenic TIMP-1 levels and the proliferative activity was further established when the TIMP-1 levels were plotted against the number of PCNA positive cells in the tumors (Figure 7B). In contrast to the Alb-TIMP-1/MMTV-MT tumors, the ‘high’ TIMP-1 expressing tumors did not differ significantly from the MMTV-MT only tumors in the number of apoptotic cells.

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**Fig. 5.** Immunohistochemical staining of TIMP-1 in tumors of (A) Alb-TIMP-1/MMTV-MT mice, (B) MMTV-TIMP-1/MMTV-MT mice, (C) MMTV-MT only littermates of A, (D) MMTV-MT only littermates of B. Original magnification, ×400.
**Fig. 6.** Proliferative activity and number of apoptotic cells are significantly decreased in Alb-TIMP-1/MMTV-MT tumors. (A) The average number of PCNA positive cells in Alb-TIMP-1/MT (n = 18) and MMTV-MT only tumors (n = 18). (B) The average number of apoptotic cells in Alb-TIMP-1/MMTV-MT (n = 24) and MMTV-MT only (n = 22) tumors. (C) Microvascular density, evaluated by CD31 immunostaining in frozen sections, in Alb-TIMP-1/MMTV-MT (n = 9) and MMTV-MT only (n = 8) tumors. The bars represent the average number of PCNA positive/40× field, apoptotic cells/40× field, and microvessels/20× field. Student’s t-test was used to calculate significance of the results (±SD).

**Fig. 7.** Positive correlation between hTIMP-1 levels and proliferative activity in ‘high’ TIMP-1 expressing MMTV-TIMP-1/MMTV-MT tumors. (A) The average number of PCNA positive cells in ‘high’ (n = 5) and ‘low’ (n = 8) expressing MMTV-TIMP-1/MMTV-MT tumors, compared with MMTV-MT only (n = 7) tumors. (B) Correlation between the number of PCNA positive cells in histological sections of the MMTV-TIMP-1/MMTV-MT tumors and hTIMP-1 levels in lysates from the same tumors. (C and D) The average number of apoptotic cells and the microvascular density was determined in ‘high’ (n = 5) and ‘low’ (n = 8) expressing MMTV-TIMP-1/MMTV-MT tumors, compared with MMTV-MT only (n = 7) tumors. The bars represent the average number of PCNA positive cells/40× field, apoptotic cells/40× field, and the number of microvessels/20× field. Student’s t-test was used to calculate significance of the results (±SD).
To further establish that over-expression of TIMP-1 in the mammary carcinomas was associated with increased proliferative activity, an in vivo BrdU study was carried out in four 13-week-old MMTV-TIMP-1/MMTV-MT and MMTV-MT only mice. The outcome of this study supported the immunohistochemical data, i.e. there were fewer BrdU positive tumor cells in the MMTV-MT only tumors (Figure 8A) than the MMTV-TIMP-1/MMTV-MT tumors (Figure 8B) with a 2-fold difference ($P < 0.05$) between the tumor groups (Figure 8C).

Like the Alb-TIMP-1/MMTV-MT tumors, the ‘high’ and ‘low’ MMTV-TIMP-1/MMTV-MT tumors showed insignificant difference in microvascular density ($P = 0.579$) from the MMTV-MT only group (Figure 7D). Similarly, CD-31 mRNA levels were not different (not shown). Western blot analysis of the angiogenic factors VEGF, bFGF and TGF-$eta$ revealed no difference in the expression level between the MMTV-MT only and the Alb-TIMP-1/MMTV-MT or the ‘high’ or ‘low’ MMTV-TIMP-1/MMTV-MT tumors (not shown). Furthermore, an angiogenesis cDNA array on pooled RNA samples from four ‘high’ TIMP-1 expressing tumors and four size matched MMTV-MT only tumors did not reveal a significant difference in expression levels of 96 angiogenesis-associated genes (not shown).

### Discussion

TIMP-mediated suppression of tumor invasion and metastases in experimental models has been attributed to their capacity to inhibit MMPs, which can collectively degrade all structural components of the ECM and are up-regulated in malignant tumors (55–57). Conversely, TIMP-1 is over-expressed in various types of human malignancies (32–46) and over-expression of TIMP-1 in breast carcinoma cells results in increased tumorigenesis (30). Discoveries of TIMP-1 as a growth factor (8–11), an inhibitor of angiogenesis (13) and a survival factor (18) have further complicated attempts to elucidate its role in carcinogenesis.

TIMP-1 is widely distributed in the body and is present in the circulation, body fluids and interstitial tissues (4,58). The reported median value of TIMP-1 plasma levels in healthy volunteers ranged widely from ~70 to 610 ng/ml (59–61). Because ELISA for mouse TIMP-1 was not available, we were unable to measure the endogenous TIMP-1 plasma levels in the transgenic mice. Instead, semi-quantitative reverse zymography was used to assess total TIMP-1 plasma levels from four size matched Alb-TIMP-1 mice and four size matched MMTV-MT mice at 6 weeks of age. In view of the data showing that transgenic TIMP-1 plasma levels were significantly higher in the Alb-TIMP-1 mice than wt littermates at 6 weeks of age.
of 400–500 ng/ml can be maintained for at least 30 weeks without adverse effects on the animals, this transgenic model is well suited for long-term studies to assess the systemic effects of biologically active TIMP-1 on various disease processes that involve excessive MMP-mediated tissue degradation, including cancer.

Chronic exposure to elevated levels of circulating transgenic TIMP-1 resulted in decreased tumor weights and lower incidence of lung metastases in the Alb-TIMP-1 mice. MMP inhibition is the TIMP-1 function that is generally implemented in tumor growth suppression (62). This is supported by our data showing significantly decreased levels of active MMP-2 and MMP-9 in the tumors of the Alb-TIMP-1/MMTV-MT mice. MMPs promote the early stages of tumor development through proteolytic degradation of the ECM, which can alter the mechanism controlling cell proliferation and promote neoplastic transformation (63,64). Elevated MMP-2 transcripts have been reported in stromal cells surrounding ductal and lobular carcinomas in situ and additionally, elevated MMP-1 transcripts were observed in invasive breast carcinomas (65).

Stromal reaction is allegedly critical for the progression of hyperplastic foci to carcinomas in DBA-induced mammary carcinomas in rats (66). Although TIMP-1 did not affect the number of early lesions in the DBA treated Alb-TIMP-1 mice, the data on decreased tumor incidence suggest that TIMP-1 prevented hyperplastic lesions from progressing to carcinomas and/or halted the growth of microscopic carcinomas. TIMP-1 may also decrease the bioavailability of growth factors, which are released from the ECM through MMP-mediated degradation (67). Extrapolating from our transgenic data to human breast cancer, it is conceivable that chronic delivery of TIMP-1 through the circulation can halt the progression of in situ and the early stages of breast cancer.

The MT antigen is a powerful oncogene, which alone can transform cells. When placed under the control of the MMTV-LTR, it induces spontaneous mammary carcinomas, which are of ductal origin (68). The main advantage of using the MMTV-MT model is that the mammary carcinomas arise synchronously and are metastatic (68). In considering the power of the MT oncogene to drive tumorigenesis, it was remarkable that a moderate increase in circulating TIMP-1 levels could significantly suppress tumor growth and the incidence of metastases in the Alb-TIMP-1/MMTV-MT mice. We attribute this to the availability of the blood-derived transgenic TIMP-1 in the tumor environment from the onset of mammary carcinogenesis. It was surprising however, that vascular density was not decreased in the MT-induced tumors of either the Alb-TIMP-1 or the MMTV-TIMP-1 mice. It is well established that TIMP-1 acts as an inhibitor in angiogenesis, both in angiogenesis assays (12–14) and in experimental tumor models (69).

Decreased neovascularization was also demonstrated in tumor allografts in the Alb-TIMP-1 model (27,70). We do not have an explanation for the inability of TIMP-1 to suppress neovascularization in the present study. Decreased expression of common angiogenic factors like VEGF, bFGF and TGFβ was ruled out in both the Alb-TIMP-1 and MMTV-TIMP-1 models. Moreover, cDNA array analysis of 96 angiogenesis-associated genes revealed an insignificant difference in gene expression between pooled RNA samples from the ‘high’ TIMP-1 expressing and the MMTV-MT only tumors. One may speculate that TIMP-1 was incapable of counteracting the powerful angiogenic forces of the MT mammary carcinogenesis model.

TIMP-1 acts as an anti-apoptotic factor in human breast epithelial cells (20,21). We found evidence to that effect in the tumor cells of the Alb-TIMP-1/MMTV-MT mammary carcinomas. A decrease in both proliferative and apoptotic activities in those tumors recapitulates the paradoxical functions of TIMP-1 (58,71). Nevertheless, the reduced tumor weights suggested that the capability of TIMP-1 to inhibit tumor cell proliferation was stronger than its anti-apoptotic/survival effects.

The growth suppressive effects of TIMP-1 in the Alb-TIMP-1 mice were not recapitulated in the MMTV-TIMP-1 mice. This was surprising when considering the large amount of transgenic TIMP-1 released from the tumors of the ‘high’ expressing carcinomas into the circulation. The ineffectiveness of the circulating TIMP-1 in suppressing tumor growth in the MMTV-TIMP-1/MMTV-MT mice may be due to the fact that high circulating TIMP-1 levels were not present at the onset of tumorigenesis. Also, the association between high TIMP-1 levels and increased proliferative activity in these tumors suggests that high intracellular TIMP-1 has growth promoting effects. This is in keeping with the reported growth promoting function of TIMP-1, which involves activation of Ras and the mitogen-activated protein kinase (MAPK) pathway (8,72). TIMP-1 is also implemented in growth promotion of human urothelial cancer where the Ki-67 labeling index was found to correlate with the TIMP-mRNA levels (73).

The lack of tumor weight gain of the ‘High’ TIMP-1 expressing tumors with increased proliferative activity has not been resolved so far and requires further studies. The outcome of the mammary carcinogenesis studies in transgenic Alb-TIMP-1 and MMTV-TIMP-1 models stresses the complex biological effects of TIMP-1 on the tumor environment. It may also reflect paradoxical functions of intra- and extracellular TIMP-1. This is in accord with findings by Goss et al. in Min mice, which develop spontaneous intestinal tumors (74). Systemic treatment of the Min mice with synthetic MMP inhibitors suppressed intestinal tumor growth, whereas overexpression of TIMP-1 in the gastrointestinal tract had either no effect or increased Min tumor multiplicity.

An imbalance in favor of MMPs is thought to be critical in promoting tumor dissemination. Although not highly significant, there was decreased incidence of lung metastases in the Alb-TIMP-1/MMTV-MT mice. This finding concurs with inhibition of intravenous metastases, following intraperitoneal administration of recombinant TIMP-1 (24). It is intriguing that the incidence of lung metastases was not decreased in the MMTV-TIMP-1/MMTV-MT mice, which displayed higher terminal TIMP-1 plasma levels than the Alb-TIMP-1/MMTV-MT mice.

In summary, the data from our transgenic models indicate that the presence of elevated TIMP-1 plasma levels from the onset of tumorigenesis is effective in suppressing mammary tumor growth and metastases. This suggests that chronic systemic TIMP-1 treatment will be beneficial in the treatment of various human diseases that involve tissue destruction from excessive MMP activities. Although synthetic MMP inhibitors have been efficient in halting tumor growth and dissemination in rodent models, they have yielded negative or borderline results in clinical trials of late stage malignancies (71,75). Further studies are required to get deeper insight into the biology of the MMPs that are crucial for the advancement of the early stages of human breast cancer. Data from such studies will be useful for the design of specific synthetic MMP
inhibitors that have fewer side effects and are more effective than the broad-spectrum MMP inhibitors used in clinical trials previously. It will be very interesting to see whether such molecules will be of therapeutic benefit in adjuvant treatment of cancer and/or as chemopreventive agents.

References
