Blocking Tumor Growth, Invasion, and Metastasis by Maspin in a Syngeneic Breast Cancer Model

Heidi Y Shi, Weiguo Zhang, Rong Liang, Shaji Abraham, Francis S. Kittrell, Daniel Medina, and Ming Zhang

ABSTRACT

Maspin is a unique serine protease inhibitor of which the down-regulation is associated with the development of breast cancers. In vitro, recombinant maspin inhibits tumor cell migration and invasion. Overexpression of maspin in transgenic mice is protective against tumor progression. Additionally, maspin acts as an angiogenesis inhibitor in rat cornea model and in a xenograft tumor model. To additionally prove that maspin is directly involved in the suppression of tumor growth and metastasis, we tested maspin in a new syngeneic mammary tumor model, TM40D. This model involves the implantation of TM40D mammary tumor cells orthotopically to the mammary gland; tumors grew within the gland and then become invasive and metastatic to other organs. Here we demonstrate that TM40D cells in implanted mammary glands are highly invasive. Overall, a 75% rate of invasion and metastasis was observed in this model. However, both primary tumor growth and metastasis were significantly blocked in TM40D cells that overexpress maspin as a consequence of plasmid or retrovirus infection. Maspin-transfected tumors tended to have tumor encapsulation and less necrosis, which were associated with better prognosis and lower invasiveness. Thus, maspin can block primary tumor growth as well as invasion and metastasis. These data support the concept that maspin has a strong protective role against tumor progression.

INTRODUCTION

Tumor metastasis involves such multiple processes such as invasion, intravasation, extravasation, and growth at a secondary site (1, 2). To study breast cancer metastasis, one needs to use suitable animal models that can mimic the process of tumorigenesis in the mammary gland and allow the tumors to become invasive and metastatic. The mammary gland is a natural site for implantation for both normal epithelial and neoplastic cells (3, 4). A frequently studied model is serial transplantation of preneoplastic mammary outgrowth lines (5, 6). These stable transplantable outgrowth lines are tumorigenic and invasive. One such mammary outgrowth line, TM40D, was found to be moderately tumorigenic, but the tumors were highly metastatic (5, 7).

Maspin is a unique member of the serpin (serine protease inhibitor) family (8) of which the down-regulation is associated with the development of breast cancers (9–14). Initial experiments demonstrated that recombinant maspin, made in bacteria, yeast, or insect, inhibits invasion and motility of mammary carcinoma cells in culture (8, 15–19). Maspin was also shown to inhibit endothelial cell motility and angiogenesis in a xenograft tumor model (20), suggesting that the antiangiogenesis property is involved in tumor suppressor activity of maspin. In vitro, maspin gene is expressed in normal cells but it is transcriptionally down-regulated in tumor cells (11, 21–23). It was also found that p53 could induce maspin expression by transcriptional activation, placing maspin as one of the few p53-targeted genes involved in tumor migration and invasion (22).

We established maspin overexpression previously in transgenic mice and crossed them with a strain of oncogenic WAP-SV40 T antigen mice to explore maspin function in vivo. We showed that overexpression of maspin in bitransgenic mice could partially block mammary tumor progression (24). The partial blockage was attributable to the fact that maspin was under a WAP promoter of which the expression was dependent on pregnancy, whereas the loss of two very potent tumor suppressors, retinoblastoma and p53, overrode the inhibition of maspin. To additionally demonstrate maspin anti-invasion and antimetastasis functions in vivo, we tested maspin gene function in the TM40D tumor model, in which TM40D cells or transfectants of maspin were implanted back into a mammary gland, and their growths and metastasis were monitored. Here we demonstrate that the TM40D mammary tumor cells in syngeneic implantation model are highly invasive. Higher tumor growth rate and extensive invasion and metastasis were observed in TM40D cells without the maspin transgene. These processes were significantly inhibited by maspin in maspin transfectants. Tumors from maspin transfectants tended to have tumor encapsulation and less necrosis, which were associated with better prognosis and lower invasiveness. These data demonstrate that maspin is capable of blocking primary tumor growth and metastasis in a syngeneic animal model.

MATERIALS AND METHODS

Cells and Cell Culture. TM40D mammary tumor cell line was established from a TM40D primary tumor that arose in serially transplanted TM40D preneoplastic outgrowth line (5, 7). TM40D cells were grown in the medium of DMEM/F12 with 5% FBS and EGF and IGF-I as described previously (5). Passage 8 cells were used in these experiments.

Establishing Maspin Transfectants in TM40D Mammary Tumor Cells. Three groups of maspin transfectants and their vector controls were established. In group 1, human maspin cDNA (1.3 kb full length) was inserted into the pEF vector by restriction enzyme digestion of EcoRI and XhoI. The maspin gene was placed under elongation factor promoter. The pEF-maspin construct and pEF control vector were transfected into TM40D cells by the method of electroporation (25). Stable clones of pEF-maspin and pEF-control were selected with 300 μg/ml G418. Maspin clones were examined by RT-PCR for the presence of human maspin mRNA and by immunostaining using anti-maspin polyclonal antibody Abs4A (9). For group 2, retroviral stable transfectants were constructed as follows: the human maspin cDNA was cloned into pS2-GFP, a retroviral vector that was derived from pS2 family of vectors (26), wherein maspin cDNA and GFP were expressed independently from 5’ long terminal repeat and pECM promoters, respectively. The plasmid constructs pS2-maspin GFP and pS2-GFP were transfected into 293T cells, along with pECo plasmid using fugeal reagent, to produce infective viral particles. The viral supernatants were then allowed to infect TM40D cells in the presence of Polybrene. The transduced cells were then selected in presence of 100 μg/ml of zeocin and sorted by flow cytometry for green fluorescence emitted by GFP. These retroviral stable transfectants were then analyzed for the presence of human maspin cDNA by RT-PCR and by immunostaining with Abs4A antibody. For group 3, mouse maspin cDNA was cloned in the pEF vector. The stable clones were established as described for group 1. To rule out the possibility of clonal variations, we randomly selected maspin-positive and control clones from three groups and used them for orthotopic implantation.

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3The abbreviations used are: RT-PCR, reverse transcription-PCR.
For RT-PCR analysis of human maspin RNA, total RNAs were isolated from control and maspin transfectants only (data not shown; 5’ primer: AATTTAAGGTGAAAAAGATG, 3’ primer: TCTATGGAATGCCCATCTTC). For immunostaining, cells were grown on chamber slides and fixed in 4% paraformaldehyde solution for 1 h. The slides were blocked with 10% normal horse serum for 1 h before they were treated with the primary antibody Abs4A at a dilution of 1:200. The secondary antibody (Texas-red conjugated goat antirabbit antibody; Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1:1000 at room temperature for 1 h. Slides were viewed under a Leica fluorescence microscope.

**Implantation of Transfectants to BALB/c Mice.** BALB/c mice were initially purchased from Harlan Sprague Dawley, Inc. and bred in our animal facility to generate offspring females 8 weeks of age. In groups 1 and 2, the sister pairs were evenly divided for the implantation of maspin clones or control clones. Tumor initiation was monitored every two days by palpation. Tumor volume was calculated using the formula: length × width²/2 (2). Tumor growth rate was monitored every 2 days by caliper measurement and tumor volume calculated as volume (mm³)/day.

In group 1, a random-selected pEF-maspin clone and a pEF control clone were used in the implantation. All of the tumor cells were grown to 70–85% of confluence before being harvested for cell counting. Each #4 mammary gland was injected with 5 × 10⁵ maspin transfectant cells or an equal number of control cells in a volume of 10 µl. Nine mice were used for maspin transfectants and nine for controls (each group contained 18 injection sites). All of the mice were sacrificed when the primary tumors grew to ~1 cm in diameter.

For group 2, random collected retrovirus transfectants from maspin and control groups were cultured in TM40D growth medium to the confluence of 70–85% and harvested for cell counting. A total of 2 × 10⁵ cells in a volume of 10 µl from each transfectant group was injected into #4 mammary gland. Thirteen mice were used for two random selected maspin transfectants (26 sites) and 12 mice for two random selected control transfectants (24 sites). All of the mice were allowed to grow ~35 days after the appearance of primary tumor.

In group 3, two random selected mouse maspin transfectants (pEF-mMaspin) and one control pEF clone were used in the implantation. Each #4 mammary gland was injected with 5 × 10⁵ cells in a volume of 10 µl. Three mice were used for control clone (6 sites) and six mice were for maspin transfectants (12 sites).

**Tumor Histology and Immunohistochemistry.** Mammary tumors were fixed in 10% neutral formalin buffer, embedded in paraffin, and sectioned at 5 µm. Sections were stained by H&E. Maspin expression was evaluated by immunostaining. Tissue sections were treated with proteinase K (10 µg/ml) for 10 min at 37°C and quenched with 0.03% of H₂O₂ in PBS for 30 min at room temperature. The slides were blocked with 10% normal horse serum for 1 h before they were treated with the primary antibody Abs4A at a dilution of 1:200. The secondary antibody (goat-antirabbit IgG; Santa Cruz Biotechnology) was used at a dilution of 1:400 at room temperature for 1 h. Slides were then rinsed and incubated with an avidin-biotin-peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA), followed by 3,3′-diaminobenzidine color development (DAB kit; Zymed Laboratories, Inc., South San Francisco, CA).

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**Fig. 1. Immunostaining of maspin transfectants and control clone.** Cells were stained by Abs4A primary antibody and followed by Texas-red conjugated secondary antibody. A, pEF-maspin cells; B, pEF-vector control cells; C, maspin-retro are retrovirus transfected stably clones; D, control-retro are retrovirus vector clones.
Statistical Analyses. Tumor-free curve was generated with Kaplan-Meier analysis using the Statview software program. Differences between groups were compared using the Student t test (two-sided analysis). A $P < 0.05$ was considered as significant.

RESULTS

In an attempt to establish a high invasive implantation tumor model, we chose the TM40D cell line from the TM (transformed mammary cells) series of mammary outgrowth. TM40D cells were initially isolated from FSK4 mammary epithelial cells that were capable of forming a hyperplastic outgrowth and tumors when implanted into mammary glands of BALB/c mice (5, 7). To test whether maspin could block tumor growth in vivo, maspin transfectants were established using TM40D cell line. Two experiments were carried out using maspin stable clones carrying either expression plasmid or clones with maspin gene integrated in the chromosome by retrovirus.

In the first experiment, two groups of paired mice were implanted at 8 weeks of age with either maspin plasmid transfectants or control transfectants in both #4 mammary glands. This group is designed to study the effect of maspin on early tumor growth and local invasion when primary tumors have relative small size and less necrosis. The second group used clones from retrovirus-transfected maspin or vector control, and tumors were allowed to grow for $>5$ weeks so that tumor invasion and distant metastasis could be monitored. Random clones from maspin plasmid transfectants and retrovirus transfectants were grown in cell culture. The expression of maspin in these clones was examined by RT-PCR analysis (data not shown) and immunostaining. The level of maspin expression was significantly higher in these maspin transfectants compared with their control vector transfectants (Fig. 1).

Tumors from both groups were implanted to #4 mammary glands of 8-week-old female BALB/c mice. As shown in Fig. 2A, the mean time

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for tumor appearance (50% of tumor end point) in the control group was 24 days, whereas it took significantly longer (36 days) for the maspin transfectants to develop palpable tumors ($P < 0.001$). When fewer TM40D cells were implanted (group 2), the mean tumor appearance period (50% of tumor end point) was 45 days for controls, whereas for maspin transfectants, it was delayed to 85 days (Fig. 2C). Implanted control TM40D cells developed palpable tumors nearly 100% in both group 1 and 2; in contrast, only 77.8% of mice implanted with maspin transfectants develop palpable tumors in group 1. This phenomenon was more evident in group 2, where 84.6% of implanted sites failed to develop tumors.

To completely rule out the possibility that the reduced tumor growth was attributable to immune reaction to human maspin used in the transfectants, an experiment (group 3) was carried out using mouse maspin stable clones and control tumors for implantation.

Fig. 3. Histology of tumor progression and immunostaining of maspin protein (group 1). A–D, histology of TM40D primary tumors was shown. A, control TM40D tumors without encapsulation; B, maspin transfectants with tumor encapsulation; C, control tumors displaying necrosis; D, lack of tumor necrosis in maspin transfected tumors. Higher expression of maspin was seen in maspin transfected tumor (F) than control (E). Photographs in A and B were $\times 100$ (bar, 200 $\mu m$). Others were $\times 200$ with a bar of 100 $\mu m$. Inset bars, 20 $\mu m$. 

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Mouse maspin also significantly inhibited tumor growth similar to its human homologue in groups 1 and 2 (Table 1). We examined the growth rate and histology of primary tumors from group 1 samples. The tumors were relatively small in size (0.5 cm$^3$) when the mice were sacrificed so that we could examine primary tumor growth rate and tumor growth patterns. Among those sites that had developed palpable tumors, the growth rates for controls were significantly higher than maspin clones in both groups 1 and 2 (See Fig. 2 and Table 1). As shown in Fig. 2B and D, control tumors grew nearly exponentially, whereas the curves for maspin clones were more flat. The tumor histology was also drastically different. In general, the presence of tumor encapsulation was associated with better prognosis, whereas the presence of excessive necrosis correlated with a more aggressive phenotype. Most sections from control TM40D tumors had necrosis but lacked tumor encapsulation (Fig. 3, B and C, and Table 2). In contrast, all of the maspin-expressing tumors had a fibrous capsule surrounding the tumors but few had necrosis (Table 2). Immunostaining with maspin antibody showed that maspin level was clearly higher in the section of maspin-transfected tumors than control tumors (Fig. 3, E and F).

To additionally examine the effect of maspin on invasion and distant metastasis, group 2 mice were sacrificed after a relatively long-term observation. On dissection of the mice, the majority of control mice had multiple tumors surrounding the intestines and some had tumors on the pleural surface, indicating these tumors had invaded through the abdominal muscle from the #4 mammary gland (Fig. 4). None of the maspin tumor-bearing mice had visible tumor formation on the intestine and pleural surfaces. To examine distant metastasis, lung tissues from group 2 mice were collected and sectioned for microscopic analysis. Two serial sections separated 100 μm were selected to score for micrometastatic tumor foci under a high-power microscope. As shown in Table 2, 33.3% (4 of 12) control mice developed lung metastasis, whereas none of the maspin-containing tumor mice had any lung metastasis. Overall, we observed that 75% of control tumor mice had either invasion or distant metastasis (9 of 12 mice). In addition, we observed local invasion of the muscle adjacent to the tumor and into blood vessels in the control TM40D tumor sections but not with maspin-containing tumors (Fig. 4).

**DISCUSSION**

Breast tumor metastasis involves both local invasion and secondary site growth. These processes are best studied in vivo in immune competent animals. There are few animal models available for the analysis of mammary invasion and metastasis. Here, we demonstrate the suitability of a syngeneic tumor implantation model for such a study. We have shown that TM40D mammary tumors, when implanted into a mammary gland, were highly invasive. Local invasion was evident in primary tumors. After long-term tumor observation, TM40D cells invaded and formed tumor foci in distant sites such as the intestine and pleural surface. They also metastasized to lung, likely through the circulation. The fact that we have observed a 75% rate of invasion and metastasis in this model makes it a very attractive system to study mammary tumor invasion and metastasis.

![Fig. 4. Tumor invasion and metastasis in TM40D model. Histology of mammary tumors invaded to intestine (A), pleural surface (B), and metastasized to lung (C). Local invasion of control tumors to mammary duct (D), muscle (E), and the blood vessel (F). Arrows, presence of tumor cells. Photographs were ×200 (bar, 100 μm).](cancerres.aacrjournals.org)
Several studies link maspin function with tumor progression. Maspin was shown to suppress invasive phenotypes of human breast cancer cells by modulating their integrin expression (12). In oral squamous cell carcinoma, high maspin expression was associated with the absence of lymph node metastasis and with better rates of overall survival (27). It was reported that breast cancer patients with high levels of maspin in their bone marrow tended to remain disease-free for longer period, whereas their low maspin controls were more likely to have recurrence (28). In this regard, maspin may be used as marker for the identification of relapse risk. Inhibition of human breast cancer by differentiation reagent peroxisome proliferator-activated receptor γ and γ linoleic acid was associated with up-regulation of the maspin gene (29, 30).

Two animal studies have implicated the direct involvement of maspin in the suppression of primary tumor and metastasis (8, 24). Partial inhibition of tumor growth was seen in nude mice, but the conclusion was compromised by the small group sizes and the use of nude mouse (8). Transgenic expression of maspin displayed partial inhibition because of the fact that maspin transgene was under a relatively weak promoter (24), and SV40 T antigen caused loss of two other potent tumor suppressors, retinoblastoma and p53 in the bitransgenic mice (31). To demonstrate without any doubt that maspin does inhibit tumor growth as well as tumor invasion and metastasis in an intact animal, we examined a new syngeneic mammary tumor model. In the first group, we examined the growth rate when tumors were relatively small and in exponential growth phase. Whereas the control tumors grew very rapidly, the maspin transfectants displayed a slow, flat growth curve. We also examined tumor growth patterns for control and maspin transfectants. Two parameters widely used by tumor biologists are tumor encapsulation and necrosis. Aggressive tumor cells have high invasiveness and are more likely to break the capsule (capsular rupture) and invade into the mammary fat pad (32). The aggressive tumors are also associated with the presence of excessive necrosis (33). As shown in Table 2, control tumors indeed had less encapsulation and more necrosis than maspin transfectants, indicating that maspin level is associated with better prognosis. This finding is in line with a previous report that higher a maspin level is correlated with better prognosis and low invasion (27, 28).

To additionally demonstrate the tumor suppressive activity of maspin and its role in invasion and metastasis, we repeated the experiment in group 2 with retrovirus-transfected maspin clones and control virus transfectants. The retrovirus maspin transfectants were used because of the possibility that some maspin plasmid transfectants might lose the plasmid without antibiotic selection in vivo. The maspin transfectants were more effective than plasmid maspin clones in inhibiting tumor development. Despite the longer time of tumor observation (40 more days than control), maspin transfectants had no visible tumor invasion and lung metastasis, whereas such invasion was widely seen in control mice.

Human maspin has 89% identity to mouse maspin (16). Because of the high homology and the unusual tolerance of the mammary gland (34, 35), we did not think an immune rejection to human maspin was the cause of tumor suppression. To definitively rule out that possibility, we carried out a third experiment using mouse maspin transfectants for implantation. Once again, mouse maspin significantly inhibited tumor growth rate, confirming that maspin itself was primarily responsible for blocking tumor progression.

In summary, we have used a new mammary tumor model to examine the role of maspin in tumor progression. Maspin by itself, when introduced in the tumor cells, can block primary tumor growth, tumor invasion, and metastasis. Thus, maspin may serve as a potential important antitumor and antimetastasis reagent in cancer therapy.

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