Inhibition of Pulmonary and Skeletal Metastasis by a Transforming Growth Factor-β Type I Receptor Kinase Inhibitor

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Abstract

Transforming growth factor-β (TGF-β) signaling has been shown to promote invasion and metastasis in various models of human cancers. In this study, we investigated the efficacy of a TGF-β type I receptor kinase inhibitor (TβRI-I) to limit early systemic metastases in an orthotopic xenograft model of lung metastasis and in an intracardiac injection model of experimental bone and lung metastasis using human breast carcinoma MDA-MB-435-F-L cells, a highly metastatic variant of human breast cancer MDA-MB-435 cells, expressing the enhanced green fluorescent protein (EGFP). Treatment of the cells with the TβRI-I had no effect on their growth but blocked TGF-β-stimulated expression of integrin αvβ3 and cell migration in vitro. Systemic administration of the TβRI-I via i.p. injection effectively reduced the number and size of the lung metastasis in both orthotopic xenograft and experimental metastasis models with no effects on primary tumor growth rate compared with controls. TβRI-I treatment also reduced the incidence of widespread early skeletal metastases in the femur, tibia, mandible, and spine detected by whole-body EGFP fluorescence imaging. Tumor burden in femora and tibiae was also reduced after TβRI-I treatment as detected by histomorphometry analysis compared with the placebo controls. Our results indicate for the first time that abrogation of TGF-β signaling by systemic administration of the TβRI-I can inhibit both early lung and bone metastasis in animal model systems and suggest antimitastatic therapeutic potential of the TβRI-I. (Cancer Res 2006; 66(13): 6714-21)

Introduction

Metastatic cancer affects millions of people worldwide, yet the vast majority of patients with cancer metastasis remain incurable (1). Identification of new therapeutic targets for metastatic cancer is paramount for improving the survival of patients with metastases.

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that plays a central role in maintaining epithelial homeostasis. In early carcinogenesis, TGF-β acts as a tumor suppressor by inhibiting cell proliferation (2–4). However, in the late stage, several studies showed that primary tumor cells could reprogram their response to TGF-β by dysregulation or mutational inactivation of various components of TGF-β signaling pathway and through cross-interaction with other oncogenic pathways. Consequently, TGF-β signal becomes metastasis promoting (5, 6). This phenomenon is well illustrated in genetically related progression models of a human mammary epithelial cell line, in which dominant-negative blockade of TGF-β signaling was shown to promote tumorigenicity of a low-grade premalignant cell but inhibited metastasis of a high-grade tumorigenic cell (7, 8). Thus, blocking TGF-β signaling especially in advanced stages of cancer may result in beneficial therapeutic responses by inhibiting metastatic progression.

TGF-β transduces its signal through two highly conserved single transmembrane serine/threonine kinase receptors, termed type I (TβRI) and type II (TβRII) receptors (3). TβRI activates TβRI on formation of the ligand-receptor complex by hyperphosphorylating serine/threonine residues in the GS region of TβRII. Activated TβRI in turn phosphorylates Smad2 and Smad3, which interact with Smad4, translocate to the nucleus, and regulate transcription of target genes. Several potential therapeutic interventions targeting the TGF-β pathway are currently in the process of development (9). Due to its central role in TGF-β signaling, TβRI is emerging as a novel target for the blockade of the tumor-promoting activity of the TGF-β pathway (10). Recently, several small molecules that inhibit TβRI kinase activity have been developed and shown to potentely inhibit TGF-β activity in vitro (10, 11). In this study, we have examined the effect of the systemic administration of an ATP-competitive TβRI kinase inhibitor (TβRI-I) on breast cancer–induced lung and bone metastasis using an orthotopic xenograft model of lung metastasis and an experimental model of bone metastasis. Our results show, for the first time, that treatment with the TβRI-I significantly inhibited tumor cell homing to the skeleton and tumor burden in the lung and bone, supporting the notion that blockade of TGF-β pathway may eventually lead to a novel therapeutic strategy for metastatic breast cancer.

Materials and Methods

Cell line. MDA-MB-435-F-L cells, a highly invasive and metastatic variant of the human breast carcinoma MDA-MB-435 cells, were isolated in our laboratory (12) and used in this study. The culture was maintained in McCoy’s 5A medium supplemented with pyruvate, vitamins, amino acids, antibiotics, and 10% fetal bovine serum (FBS) as described previously (13). MCF-7 and MCF-10A cell lines were originally obtained from Michigan Cancer Foundation (Detroit, MI). The culture medium for MDA-MB-435-F-L cells, a DMEM-F12 medium as described previously (14).

Animals. Four- to 5-week-old female athymic nude mice (obtained from Harlan Sprague Dawley, Inc., Indianapolis, IN) were used for in vivo animal experiments. The animals were housed under specific pathogen-free conditions. All animal protocols were approved and monitored by the Institutional Animal Care and Use Committee.

Bioassays of TβRI-I. The TβRI-I used in our study was reported previously to be an ATP-competitive inhibitor of the TβRI kinase (15, 16).
were from Cell Signaling Technology (Beverly, MA), antibody to integrin or absence of TGF-
expression construct. At 3 hours after transfection, cells were treated with
activity normalized with
h
3 and varying concentrations of the T
RI-I for 20 hours. Luciferase activity normalized with β-gal activity in the cell lysates was then determined.

Western blotting. The MCF-7 and MDA-MB-435-F-L breast cancer cells were treated with different concentrations of the T
RI-I in the presence or absence of TGF-β3, and the cell lysates were used in Western blotting analysis as described previously (18). Antibody to the phosphorylated Smad2 was from Upstate Biotechnology (Charlottesville, VA), antibodies to the phosphorylated extracellular signal-regulated kinase (ERK) 1/2, p38, and AKT were from Cell Signaling Technology (Beverly, MA), antibody to integrin β3 was from Cell Signaling Technology, and antibodies to integrin αv, p150
, and c-Myc were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell proliferation assay. The MDA-MB-435 and MCF-10A cells were plated in a 96-well plate at 2,000 cells per well in the presence of varying concentrations of the T
RI-I. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done to obtain relative cell number after 5 days of incubation as described previously (19).

In vitro cell migration. Cell migration assays were done using 24-well Boyden chambers with 8-μm pore polycarbonate membranes (Becton Dickinson Labware, Rockville, MD). In the first assay, MDA-MB-435-F-L cells were seeded in the upper chamber at 50,000 cells per well in a serum-free medium and treated with 1 ng/mL TGF-β3 in the presence or absence of 100 nmol/L T
RI-I. In another assay, cells in a serum-free medium were pretreated with the antibody to integrin αv, and β3 subunits or with a control IgG at 1:200 dilution for 30 minutes at 4°C. Cells were then seeded in the upper chamber at 50,000 cells per well and treated with 1 ng/mL of TGF-β3. The lower chamber contained 0.8 mL medium with 10% FBS as chemoattractant. After 48 hours of incubation, cells were removed from the upper surface of the invasion chamber with a cotton swab, and the cells that migrated to the lower surface of the invasion chamber were stained with 0.5% crystal violet for 30 minutes. Cells were washed three times with PBS and counted in a phase microscope (20).

Spontaneous in vivo lung metastasis assay. The enhanced green fluorescent protein (EGFP)-expressing MDA-MB-435-F-L cells were harvested in the upper chamber at 50,000 cells per well in a serum-free medium and treated with 1 ng/mL TGF-β3 in the presence or absence of 100 nmol/L T
RI-I. In another assay, cells in a serum-free medium were pretreated with the antibody to integrin αv, and β3 subunits or with a control IgG at 1:200 dilution for 30 minutes at 4°C. Cells were then seeded in the upper chamber at 50,000 cells per well and treated with 1 ng/mL of TGF-β3. The lower chamber contained 0.8 mL medium with 10% FBS as chemotacticant. After 48 hours of incubation, cells were removed from the upper surface of the invasion chamber with a cotton swab, and the cells that migrated to the lower surface of the invasion chamber were stained with 0.5% crystal violet for 30 minutes. Cells were washed three times with PBS and counted in a phase microscope (20).

Results

In this study, we have used the highly metastatic MDA-MB-435-F-L human breast cancer cell line, which has been shown to induce both pulmonary and widespread skeletal metastases in nude mice (12), to evaluate the potential of T
RI-I in blocking the early invasion and subsequent development of lung and bone metastatic tumors.

Blockade of TGF-β signaling by T
RI-I. The T
RI-I used in the current study was initially described by two reports as a highly efficacious and specific inhibitor of T
RI (15, 16). To confirm the TGF-β antagonistic activity of the T
RI-I synthesized by us, several assays were done. The compound was found to inhibit the PAI-1 promoter activity induced by TGF-β in a dose-dependent manner with an IC50 of ~4 nmol/L (Fig. 1A), which is similar to the previously reported IC50 (15, 16). It also significantly inhibited the activity of the transcriptional activity of a constitutively active T
RI (data not shown). Because Smad2 is a substrate of RI kinase, we next determined the inhibitory activity of the compound in TGF-β-induced phosphorylation of Smad2. As shown in Fig. 1B, the compound effectively blocked TGF-β-induced Smad2 phosphorylation in MCF-7 cells in a dose-dependent manner, whereas it showed no effect on the phosphorylation of other intracellular proteins, such as ERK, p38, and AKT, indicating its specific inhibitory effect on T
RI. Similar inhibition of Smad2 phosphorylation by the T
RI-I was also observed in MDA-MB-435-F-L cells (Fig. 1C). In addition, T
RI-I inhibited TGF-β-mediated induction of a TGF-β-responsive promoter activity in the MDA-MB-435-F-L cells in a dose-dependent manner (Fig. 1D). These results confirm that the T
RI-I can specifically and effectively block TGF-β signaling in MDA-MB-435-F-L cells.

MDA-MB-435-F-L cells were resistant to the growth inhibition by TGF-β. TGF-β suppresses carcinogenesis mainly by
However, they are not growth inhibited by the treatment with TβRI-I not only attenuated the growth inhibition by the exogenous TGF-β but also stimulated the growth of MCF-10A cells in the absence of TGF-β (Fig. 2A), suggesting that the growth of MCF-10A cells was also inhibited by autocrine TGF-β. On the other hand, the treatment with TβRI-I at 100 nmol/L had little effect on the growth of MDA-MB-435-F-L cells in the presence or absence of TGF-β (Fig. 2A). In fact, treatment with TβRI-I at concentrations up to 500 nmol/L did not show any effect on the growth of MDA-MB-435-F-L cells (Fig. 2B), consistent with the observation that TβRI-I treatment did not affect the primary tumor growth of the cells in vivo as described below. The insensitivity of the MDA-MB-435-F-L cells to the growth-inhibitory activity of TGF-β was explained by the fact that the cells do not express p15ink4b (data not shown), and TGF-β treatment showed no effect on their expression of p15ink4b and c-Myc (Fig. 2C). The inability of TGF-β to inhibit c-Myc expression was also observed in another human breast carcinoma cell line MDA-MB-231 (21). Therefore, like many other carcinoma cell lines, MDA-MB-435 cells retain TGF-β signaling but lose the growth response to TGF-β, such that TGF-β signaling becomes tumor promoting.

**TβRI-I inhibited TGF-β-mediated cell motility.** Tumor cell motility is an integral process in the process of invasion and metastasis. One potential mechanism by which TGF-β promotes tumor metastasis is to enhance tumor cell motility. We tested the effect of the TβRI-I on the TGF-β-induced migration of MDA-MB-435-F-L cells initially in a wound closure assay. The treatment inhibiting cell proliferation through the induction of the expression of cyclin-dependent kinase inhibitor, p21cip1 and p15ink4b, and the stimulation of the expression of c-Myc. However, many aggressive carcinoma cells are often resistant to the growth-inhibitory activity of TGF-β, although they retain an operational TGF-β signaling pathway (4). MDA-MB-435-F-L cells are responsive to TGF-β with respect to its gene transcription regulation as shown in Fig. 1D. However, they are not growth inhibited by the treatment with TGF-β as shown in Fig. 2A. In contrast, the growth of the untransformed human mammary epithelial MCF-10A cells was inhibited by TGF-β in a dose-dependent manner (Fig. 2A). Treatment with TβRI-I not only attenuated the growth inhibition of TGF-β. TβRI-I at various concentrations was transiently transfected with pSBE4-Luc and a reporter construct, were analyzed for luciferase activity. At 3 hours after transfection, cells were treated with TGF-β3 and 100 nmol/L of TGF-β1. After 16 hours of incubation, cells were lysed and the lysate was analyzed for luciferase activity.

**Figure 1.** Inhibition of TGF-β signaling by TβRI-I. A, mink lung epithelial cells, stably transfected with a PAI-1 promoter-luciferase reporter construct, were plated in a 96-well plate and treated with 0.4 ng/mL TGF-β3 and various concentrations of the TβRI-I. After 16 hours of incubation, cells were lysed and the lysate was analyzed for luciferase activity. Points, mean from three different wells; bars, SE. B, MCF-10A breast cancer cells were treated with or without TGF-β3 at 0.5 ng/mL and various concentrations of the TβRI-I for 24 hours. Western blotting was done with the cell lysates for activated/phosphorylated Smad2 (P-Smad2), ERK (P-ERK), p38 (P-p38), and AKT (P-Akt) with their respective antibodies. C, MDA-MB-435-F-L cells were treated with or without the TβRI-I at various concentrations for 2 hours before the treatment with TGF-β3 at 0.5 ng/mL for 1 hour. Western blot analysis of cell lysates was done with antibody to activated/phosphorylated Smad2, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were used to indicate equal sample loading. D, MDA-MB-435-F-L cells were transiently transfected with pSBE4-Luc and a β-gal expression construct. At 3 hours after transfection, cells were treated with TGF-β3 and various concentrations of the TβRI-I for 20 hours. Luciferase and β-gal activities were determined. Luciferase activity normalized with β-gal activity. Columns, mean from three measurements; bars, SE.
of the cells with TGF-β-stimulated cell motility, which was noticeably inhibited with the addition of the TβRI-I in a dose-dependent manner (data not shown). We then used a Boyden chamber assay to quantify the effect of TβRI-I on TGF-β-induced migration. TGF-β-induced migration of the cells was inhibited by TβRI-I as shown in Fig. 3A. Because cell migration involves the interaction between cell surface integrins and extracellular matrix (ECM) and TGF-β is known to stimulate the expression of various integrin receptors (22), we examined the effect of TGF-β and TβRI-I on the protein expression of integrin αvβ3, which is known to promote adhesion and migration of several breast carcinoma cell lines, including MDA-MB-435 cell line, which expresses a substantial level of integrin αvβ3 compared with other cell lines (23). As shown in Fig. 3B, TGF-β treatment stimulated the levels of both integrin αv and β3 subunits, whereas the addition of TβRI-I attenuated the effect of TGF-β. To determine whether TGF-β-induced integrin αvβ3 contributes to the migration of MDA-MB-435-F-L cells, we tested whether the treatment of the cells with antibodies to integrin αv and β3 subunits could inhibit TGF-β-induced migration in the Boyden chamber assay. A significant decrease of the migration of MDA-MB-435-F-L cells following treatment with antibodies to integrin αv and β3 subunits was observed compared with the treatment with a control IgG (Fig. 3C). Thus, abrogation of TGF-β signaling by TβRI-I in MDA-MB-435-F-L cells has the ability to block both TGF-β-induced integrin αvβ3 expression and, consequently, cell migration.

Systemic administration of the TβRI-I suppressed lung metastasis but did not affect primary tumor growth in an orthotopic xenograft model. To determine the effect of the TβRI-I on the tumorigenicity and spontaneous lung metastasis, we administered the TβRI-I into nude mice bearing EGFP-expressing MDA-MB-435-F-L orthotopic xenograft. To determine the safe, nontoxic dosages of the TβRI-I for daily administration, we did a short-term acute toxicity study, in which daily i.p. administration of 20 to 100 μg/mouse of the TβRI-I for 8 days did not cause any abnormal behavior, change in skin color, loss of body weight, or liver toxicity (as indicated by serum alanine aminotransferase activity) in 4- to 5-week-old female nude mice (data not shown). Therefore, we did i.p. administration of the TβRI-I at 20 μg/mouse into female nude mice bearing orthotopic MDA-MB-435-F-L/EGFP tumors of ~6 mm in diameter and found that the treatment with the TβRI-I did not produce any difference in tumor growth rate compared with the placebo group (data not shown). The experiment was terminated after 21 days of the treatment, and early lung metastasis in the treatment group and the placebo group was determined. Because the MDA-MB-435-F-L cells used in this study were labeled with the EGFP, we were able to observe and count green micrometastatic colonies in the whole lung under an inverted fluorescence microscope. Although the overall lung metastasis incidences were similar (five of five in the placebo group versus four of five in the TβRI-I-treated group), the treatment with the TβRI-I markedly reduced the number of lung metastatic colonies as shown in Table 1A. For example, four of five mice had >50 lung metastatic colonies in the placebo group, whereas none in the TβRI-I-treated mice had >50 lung metastatic colonies. Conversely, most of the TβRI-I-treated mice had <20 colonies, whereas there was no mouse with <20 colonies in the placebo group. Furthermore, most of the colonies in the TβRI-I-treated group of animals were very small (~20 μm) and appeared to be single cell invasions rather than the formation of multiple cell colonies (>100 μm) found in the lungs of the placebo group of animals. Thus, our results indicate that systemic administration of the TβRI-I can inhibit spontaneous lung metastasis of MDA-MB-435-F-L cells.

Systemic administration of TβRI-I suppressed tumor cell invasion and metastasis into the bone and lung in an experimental metastasis model. To investigate the effect of the TβRI-I on the ability of tumor cells to invade and metastasize into the skeleton, we used an intracardiac injection model of experimental metastasis. The animals were inoculated with MDA-MB-435-F-L/EGFP cells via the left ventricle of the heart and treated with the TβRI-I systemically via i.p. injection in two separate experiments. In the first experiment (study-1), the TβRI-I was given i.p. at 20 μg/mouse every other day for 34 days, and in the second experiment (study-2), it was given i.p. at 5 or 20 μg/mouse every other day for 30 days. In both experiments, the treatment with the TβRI-I started on the day of tumor cell inoculation. Whole-mouse EGFP imaging revealed that the treatment with the TβRI-I reduced the incidence of skeletal
metastasis in femur/tibia, spine, and mandible in both study-1 (Fig. 4A) and study-2 (Fig. 4B). The reduction of the metastasis incidence was statistically significant ($P = 0.005-0.029$), with an exact Pearson $\chi^2$ test in some cases. Interestingly, the two dosages of the T$_{\beta}$RI-I in study-2 produced similar inhibition, suggesting that the lower dosage of 5 µg/mouse might be optimal in this model system. Another observation we made was that, in the mice with spinal bone metastasis, the metastatic lesions were more extensive in the control mice than in the T$_{\beta}$RI-I-treated mice as assessed by the whole-mouse fluorescence imaging (Fig. 4C) and with histologic staining (Fig. 5A). The metastasis to spinal bone caused deformity of the spine as observed in X-ray radiographs, such that more control mice showed spine deformity than the T$_{\beta}$RI-I-treated mice (data not shown). Histologic staining also confirmed our findings that administration of the T$_{\beta}$RI-I reduced the tumor burden in femora and tibiae below as well as above the growth plate (Fig. 5B). Histomorphometric analysis of the femora and tibiae in the cancerous region of long bones (below the growth plate) indicated that tumor burden in all four long bones was consistently lower in the T$_{\beta}$RI-I-treated animals than in the control animals (Fig. 5C), although the differences were not statistically significant. The histologic analysis also revealed that the metastatic incidence above the growth plates in the four long bones was also lower in the T$_{\beta}$RI-I-treated animals than in the placebo-treated animals, and the difference was statistically significant with a Fisher's exact test in some bones (Fig. 5D). Thus, our results from two independent experiments indicate that, in addition to the inhibition of the number of metastatic colonies, systemic T$_{\beta}$RI-I treatment also seemed to limit metastatic tumor growth (i.e., size of metastases). Bone metastases of the MDA-MB-435 cells are weakly osteolytic, as we have observed occasionally and has been reported by others (24, 25). In the current study, we did not detect any major resorption of the trabecular and cortical bones after 5 weeks of tumor cell inoculation with both histomorphometry and radiographic analysis in both placebo and experimental groups (data not shown). Consequently, the effect of the treatment with the T$_{\beta}$RI-I on tumor cell-induced bone resorption could not be evaluated with this model system.

In addition to skeletal metastasis, intracardiac inoculation of the MDA-MB-435-F-L/EGFP cells also led to lung metastasis. Similar to the observations in the spontaneous lung metastasis after orthotopic inoculation, the systemic treatment with the T$_{\beta}$RI-I also markedly reduced the extent of lung metastasis as shown in Table 1.

### Table 1. Inhibition of spontaneous and experimental lung metastasis after systemic administration of T$_{\beta}$RI-I

<table>
<thead>
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<th>Treatment</th>
<th>Overall incidence</th>
<th>No. colonies</th>
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<td>&gt;50</td>
<td>20-50</td>
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<tr>
<td>Placebo</td>
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<td>T$_{\beta}$RI-I (20 µg)</td>
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#### A. Spontaneous lung metastasis detected by GFP fluorescence in tumor xenograft model

<table>
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<th>Treatment</th>
<th>Overall incidence</th>
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<td></td>
<td>&gt;100</td>
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<tr>
<td>Placebo</td>
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<td>5/8</td>
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<tr>
<td>T$_{\beta}$RI-I (20 µg)</td>
<td>7/8</td>
<td>0/8</td>
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<tr>
<td>Study-2</td>
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<td>T$_{\beta}$RI-I (5 µg)</td>
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#### B. Experimental lung metastasis detected by GFP fluorescence in intracardiac injection model

**Figure 4.** Decrease of skeletal metastasis incidence by T$_{\beta}$RI-I treatment in an intracardiac tumor cell injection model detected by whole-mouse EGFP imaging. Exponential growing, EGFP-expressing MDA-MB-435-F-L cells were injected into the left cardiac ventricle at 10$^5$ cells per mouse. The mice were treated with T$_{\beta}$RI-I every other day at 5 µg/mouse (T$_{\beta}$RI-I 5) or 20 µg/mouse (T$_{\beta}$RI-I 20). Metastasis to skeleton was detected with whole-body green fluorescence imaging. Treatment with the T$_{\beta}$RI-I reduced the incidence of skeletal metastasis to femur/tibia, spine, and mandible compared with the placebo control in both study-1 (A) and study-2 (B). *, statistically significant difference between the control and T$_{\beta}$RI-I groups with an exact Pearson $\chi^2$ test ($P = 0.005-0.029$). B, no spinal metastasis was observed in study-2 at 20 µg dosage. C, whole-body green fluorescence images of a representative mouse from the control and the T$_{\beta}$RI-I-treated group showing green fluorescence metastatic lesions in the femur/tibia and spine.
of the femora and tibiae was recorded and expressed as percentage tumor whereas the majority of the T
h
of nine bones; bars, SE. *D, presence of metastatic tumors above the growth plate of the femora and tibia was measured and expressed as percentage tumor incidence. *P = 0.003-0.015).

Figure 5. Inhibition of skeletal metastatic tumor burden by the treatment with the T\(\beta\)RI-I detected with histologic analyses. At the termination of the experiment, bone tissues were fixed in formalin, decalcified in 10% EDTA, and embedded in paraffin. Sections were stained with H&E, orange G, and phloxine. A, representative sections of spine are presented from a control mouse with >90% bone marrow filled with tumor cells and from a T\(\beta\)RI-I-treated mouse with <5% bone marrow filled with tumor cells. B, representative sections of a distal femur are presented from a control mouse with bone marrow filled with tumor cells and from a T\(\beta\)RI-I-treated mouse with <20% bone marrow filled with tumor cells. C, tumor area below the growth plate in the distal femur and proximal tibia was measured for each section and presented as the percentage of the total area measured through histomorphometric analysis. Columns, mean of nine bones; bars, SE. D, presence of metastatic tumors above the growth plate of the femora and tibia was recorded and expressed as percentage tumor incidence. *P = 0.003-0.015).

shown in Table 1B. Although the overall lung metastasis incidence was only slightly decreased with the treatment of the T\(\beta\)RI-I, the majority of the control mice had >100 lung metastatic colonies, whereas the majority of the T\(\beta\)RI-I-treated mice had <20 lung metastatic colonies as observed in both study-1 and study-2 (Table 1B). Therefore, our results from both experimental and spontaneous lung metastasis models indicate that the systemic treatment with the T\(\beta\)RI-I can reduce metastatic tumor burden in the lung.

Discussion

The induction and/or activation of TGF-\(\beta\) in advanced cancers have been shown to accelerate metastatic progression in various human cancer cells and animal models (4–6). Breast cancer metastasizes to multiple sites and most frequently to lung (26) and bone tissues (27), resulting in poor prognosis. In this study, we have evaluated the effect of T\(\beta\)RI-I on the tumor cell invasion and metastasis to lung and bone in a spontaneous lung metastasis and an experimental bone and lung metastasis model of human breast cancer.

We used a variant of the human breast carcinoma MDA-MB-435 cell line, termed MDA-MB-435-F-L, for our study. This variant was shown to induce extensive lung and skeletal metastasis in athymic nude mice (12). MDA-MB-435 cells were isolated from a breast cancer patient (28) and shown by several laboratories to secrete and express breast epithelium-specific proteins and milk lipid markers (28–30). Our results indicate that the MDA-MB-435-F-L cell possesses an operational TGF-\(\beta\)-stimulated promoter activity and TGF-\(\beta\)-induced Smad2 phosphorylation. On the other hand, TGF-\(\beta\) does not inhibit the growth of MDA-MB-435-F-L cells as shown in our study. As such, its signaling in the cells is likely to promote malignant progression.

Acquisition of tumor cell motility plays a fundamental role in the onset and progression of metastatic cancer, which is induced by TGF-\(\beta\) in many experimental models (31–33). The stimulation of cell motility by TGF-\(\beta\) is directly associated with its ability to enhance the expression of ECM proteins and their cell surface receptors called integrins. The expression of integrin \(\alpha_v\beta_3\) is associated with both lung and bone metastatic potential of MDA-MB-435 breast carcinoma (24, 34). Integrin \(\alpha_v\beta_3\) has also been implicated in breast cancer homing to bone in a clinical study in which the expression of \(\alpha_v\beta_3\) integrin was shown to be higher in metastatic breast cancer cells within bone than those in primary breast adenocarcinoma (35). Our study shows that blockade of TGF-\(\beta\) signaling by the T\(\beta\)RI-I can effectively block the TGF-\(\beta\)-induced invasion and metastasis.

Isolated lung metastasis occurs in 10% to 20% of women with breast cancer (36), and 60% to 74% of patients who die of breast carcinoma have pulmonary metastasis (37). In several animal models, TGF-\(\beta\) has been shown to promote lung metastasis (31, 38). The conditional induction of TGF-\(\beta\) in hosts with established TGF-\(\beta\)-responsive cancers could rapidly increase lung metastasis in a transgenic mouse model (33). In a Neu-induced transgenic breast cancer model, it was observed that TGF-\(\beta\) signaling increased the subsequent formation of lung metastasis by enhancing the extravasation of breast cancer cells into the lung parenchyma (39). Thus, aberrant up-regulation of TGF-\(\beta\) production and signaling, which are often observed in the microenvironment of a carcinoma, can promote lung metastasis. In our study, we have observed that systemic administration of the T\(\beta\)RI-I in both mouse orthotopic xenograft model and intracardiac injection model of MDA-MB-435-F-L cells reduced the number and the size of both spontaneous and experimental lung metastasis colonies compared with the placebo group. According to a retrospective study of breast cancer, longer disease-free interval from the diagnosis of a primary tumor to the detection of lung metastasis
and smaller size of pulmonary metastasis are significantly associated with an increased chance of survival (36). Thus, our results indicate that TβRI-I treatment may be effective in limiting the development and progression of breast cancer-induced pulmonary metastasis, leading to an improved survival. Our observations are consistent with a recent animal study, showing that stable expression of a Smad-binding defective type I receptor mutant in a high-grade MCF-10A-derived breast cancer cell line can significantly reduce the size of the lung metastasis foci following tail vein inoculation. Interestingly, the report also showed that the abrogation of Smad signaling enhanced tumorigenicity of a low-grade MCF-10A-derived breast cancer cell line in a xenograft model (40). This is presumably due to the attenuation of TGF-β-induced growth inhibition by the expression of the mutant RI. Because the growth of the MDA-MB-35-F-L cells is not altered by TGF-β, or by the TβRI-I in our current study, it is not surprising that the treatment with the TβRI-I showed no effect on the growth rate of MDA-MB-35-F-L xenografts.

Breast cancer metastasizes to bone in >80% of patients with advanced disease. In a recent clinical study, the presence of isolated tumor cells in the bone marrow at the time of diagnosis of breast cancer is associated with a poor prognosis (41). Tumor growth at the bone site can be extremely painful due to both the presence of the tumor mass in the bone marrow cavity as well as nerve compression. The subsequent loss of bone can lead to debilitating fractures particularly of the hip and spine (42). TGF-β signaling has been implicated in the promotion of breast cancer-induced bone metastasis in several studies (43, 44). In this study, we have evaluated the effect of systemic administration of a TβRI-I on the invasion and metastasis of MDA-MB-453-F-L cells to bones in an intracardiac injection model of experimental bone metastasis. In this model, we observed widespread skeletal metastasis, such as in femur, tibia, mandible, and spine, in the placebo controls, whereas TβRI-I treatment significantly reduced both the incidence and the extent of bone metastasis detected by whole-mouse imaging and histologic analyses. Our observations are consistent with a recent report showing that treatment with TβRI-I attenuated the invasiveness of human glioma cells in vitro and prolonged the survival of the mice inoculated intracranially with the glioma cells in vivo (45). One possible primary mechanism for the reduced incidence of bone metastasis by TβRI-I treatment as observed in our current study may be due to the inhibition of TGF-β-induced extravasation of the tumor cells into the bone marrow, similar to the TGF-β-induced extravasation in the pulmonary metastasis model as mentioned earlier (39). A recent clinical study indicated that disseminated tumor cells in the bone marrow, 3 years after diagnosis in disease-free breast cancer patients, are associated with a poor clinical outcome (46). Thus, the significantly reduced incidence of tumor cell invasion into the bone by TβRI-I treatment indicates that this approach may have potential use in the prevention of early micrometastasis of tumor cells into the bone tissues of breast cancer patients.

Although the MDA-MB-435-F-L cell is an excellent model for studying human breast cancer invasion and metastasis, it does not cause extensive, readily detectable osteolysis in the bone (12, 24, 25). Consequently, we did not detect any major loss of trabecular and cortical bones in the presence or absence of the TβRI-I treatment in the current study. Because TGF-β signaling is known to promote osteolytic bone metastasis by the human breast carcinoma MDA-MB-231 cells (47), one would expect that systemic treatment with TGF-β antagonists will also inhibit osteolytic metastasis. Indeed, our unpublished preliminary data seem to confirm this prediction in an intracardiac experimental model of bone metastasis using the MDA-MB-231 cells.

Our results indicate for the first time that abrogation of TGF-β signaling by the systemic administration of TβRI-I can effectively inhibit the invasion and colonization by the human breast cancer MDA-MB-435 cells in the lung and bone tissues. Clearly, more studies are needed for the determination of whether TGF-β antagonists, including TβRI-I, may be suitable as novel therapeutic agents for the treatment and prevention of breast cancer-induced lung and bone metastasis. Because tumor-associated overproduction of active TGF-β isoforms can attenuate host immune defense against tumor cells (48), our study using the immunodeficient nude mice did not address whether the systemic treatment with TβRI-I could also enhance host immune response to tumor cells. Therefore, it will be necessary in future studies to determine the effect of TβRI-I administration on host immune system and whether the metastasis-suppressing activity of TβRI-I can be augmented in immune proficient animal models.

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References


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