ABSTRACT

We have demonstrated previously that ectopic expression of a soluble betaglycan, also known as transforming growth factor (TGF) β type III receptor, can suppress the malignant properties of human carcinoma cells by antagonizing the tumor-promoting activity of TGF-β (A. Bandypadhyay et al., Cancer Res., 59: 5041–5046, 1999). In the current study, we investigated the potential therapeutic utility of a recombinant preparation of human and rat soluble betaglycan (sBG). Purified recombinant human sBG showed similar properties to its rat counterpart (M. M. Vilchis-Landeros et al., Biochem J., 355: 215–222, 2001). It bound TGF-β with high affinity and isoform selectivity and neutralized the activity of TGF-β1 in two bioassays. Peritumoral (50 μg/tumor, twice a week) or i.p. (100 μg/animal, every alternate day) injection of sBG into human breast carcinoma MDA-MB-231 xenograft-bearing athymic nude mice significantly inhibited the tumor growth. The administration of sBG also reduced metastatic incidence and colonies in the lungs. The tumor-inhibitory activity of sBG was found to be associated with the inhibition of angiogenesis. Systemic sBG treatment significantly reduced tumor micro vessel density detected with histological analyses and CD-31 immunostaining, as well as tumor blood volume measured with hemoglobin content. In an in vitro angiogenesis assay, treatment with the recombinant sBG significantly reduced the ability of human dermal microvascular endothelial cells to form a capillary tube-like structure on Matrigel. These findings support the conclusion that sBG treatment suppresses tumor growth and metastasis, at least in part by inhibiting angiogenesis. As such, it could be a useful therapeutic agent to antagonize the tumor-promoting activity of TGF-β.

INTRODUCTION

TGF-β is a potent regulator of cell proliferation, differentiation, extracellular matrix formation, and immune response (1). Alterations in the production of TGF-β ligand have been linked to numerous disease states including osteoporosis, hypertension, atherosclerosis, hepatic cirrhosis, and fibrotic disease of the kidney, liver, and lung (2). Increased expression of TGF-β isoforms has also been overwhelmingly shown to be associated with neoplastic development and progression in breast, colon, prostate, bladder, and gastric cancers and osteosarcomas (3). Many of these studies also indicated that the increased TGF-β production was associated with poor pathological or clinical outcomes including shorter survival time (3). Furthermore, ectopic expression of TGF-β was shown to promote tumor progression in various tumor model systems (4–6). Therefore, it is evident that TGF-β antagonists may be of potential therapeutic utility to prevent its deleterious disease-promoting effects.

Betaglycan, also known as TGF-β type III receptor, has two TGF-β binding sites (7–9) and binds all three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) with high affinity (10). A sBG consisting of its extracellular domain has been shown to bind and neutralize the activity of TGF-β isoforms with high potency in vitro (7, 11). We have shown previously that ectopic expression of the sBG can suppress the malignant properties of human carcinoma cells by antagonizing the tumor-promoting activity of TGF-β in vivo (12). In the current study, we investigated the effectiveness of a recombinant sBG in suppressing malignant progression in a human breast carcinoma xenograft-bearing nude mouse model. We administered sBG by either p.t. or i.p. injection into mice with growing tumors and observed a significant reduction of the tumor growth rate, spontaneous metastasis, and tumor vascularization. Our study demonstrates the potential therapeutic utility of sBG as a TGF-β antagonizing agent for the treatment of cancer.

MATERIALS AND METHODS

Animals. Female athymic nude mice (purchased from Harlan Sprague Dawley, Inc., Indianapolis, IN), 4 weeks of age, were used for in vivo animal experiments. The animals were housed under pathogen-free conditions.

Cell Lines. Human breast cancer cell line MDA-MB-231 and mink lung epithelial cell line CCL64 were originally obtained from the American Type Culture Collection. To determine the effect of sBG administration on the early metastatic potential of the MDA-MB-231 cells, we stably transfected the enhanced GFP expression plasmid, pEGFP-N1 (Clontech Laboratories, Inc.), into MDA-MB-231 cells and obtained a pool of GFP-expressing cells called MDA-MB-231/GFP. The expression of GFP allowed us to detect micrometastatic colonies in the whole lungs under an inverted fluorescence microscope as others have reported (13). These cell lines were cultured in McCoy’s 5A medium supplemented with pyruvate, vitamins, amino acids, antibiotics, and 10% fetal bovine serum (14). HDMECs and the culture medium EGM-2MV were obtained from BioWhittaker (San Diego, CA). Working cultures were maintained at 37°C in a humidified incubator with 5% CO2.

Preparation of Recombinant sBG. The procedures to generate, express, and purify the baculoviral recombinant human sBG were similar to those described earlier for the rat sBG (7, 11). Briefly, the human BG cDNA (15) was engineered to contain after Asp781 (the last residue of the predicted extracellular domain) a hexa-histidine tail followed by a stop codon. The mutated cDNA was used to generate a high titer recombinant baculovirus, which was used to infect High Five cells (Invitrogen). After 2 days of infection, human sBG was purified from the conditioned medium using immobilized metal-ion affinity chromatography.

Affinity Labeling in Solution and Competition Assay. TGF-β1 affinity labeling in solution and TGF-β competition assays were done as described previously (11). Briefly, 10 ng of human sBG and 100 pmol of 125-I-labeled TGF-β1 (in the absence or presence of the indicated concentrations of nonradioactive TGF-β1) were incubated for 3 h at 4°C in PBS supplemented with 0.05% (v/v) Triton X-100. Cross-linking was started by the addition of 0.1 mg/ml disuccinimidyl suberate (Pierce) and stopped after 15 min by the addition of Tris-Cl (pH 7.5) to a final concentration of 10 mM. The reaction mixture was immunoprecipitated with an antihuman sBG polyclonal antibody raised from rabbits following standard immunization protocols (16). The precipitated proteins were separated by SDS-PAGE and the 125-I-labeled TGF-β-complexed sBG was revealed by scanning in a PhosphorImager (Molecular Dynamics, Inc.). Quantitative densitometry of radiolabeled sBG was carried
out using the ImageQuant software; data were analyzed using the Prism software.

Bioassays of TGF-β1 Neutralizing Activity of Recombinant sBG. Two bioassays were used to confirm that sBG can effectively antagonize the activity of TGF-β1. In the first assay, sBG was used to neutralize the activity of TGF-β1 in stimulating the promoter activity of the human PAI-1. We used a mink lung epithelial cell line that was stably transfected with a PAI-1 promoter-luciferase construct as described by Abe et al. (17). The cells were plated in a 96-well plate at 1000 cells/well and incubated for 3 days for them to reach the exponential growth phase. They were then treated with various concentrations of TGF-β1 in the presence or absence of sBG at 5 μg/ml. After 16 h of incubation, cells were lysed, and the cell lysate was analyzed for luciferase activity. A second bioassay was used to test the ability of sBG in neutralizing TGF-β1-mediated growth-inhibitory activity in the mink lung epithelial cells. The cells were plated in a 96-well plate at 2000 cells/well and incubated with 0.05 or 0.25 ng/ml of TGF-β1 in the presence or absence of 5 μg/ml sBG. After 5 days of incubation, the relative cell number in each well was obtained with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (18) and expressed as absorbance.

In Vivo Tumor Growth and Metastasis Studies. The MDA-MB-231/GFP cells were harvested from exponential cultures and inoculated s.c. at 3 × 10^6 cells/inoculum in both sides of the inguinal mammary fat pad area of female athymic nude mice, 4 weeks of age. When the tumors grew with an average diameter of >4 mm after ~5 weeks, animals were ranked according to the tumor volume and divided into two groups such that the mean and median of tumor volume of the two groups were closely matched. In the first experiment, the mice in the experimental group were injected twice a week peritumorally (next to a tumor) with a rat recombinant sBG at a dosage of 50 μg/tumor in a total volume of 0.05 ml of PBS. The mice in the control (placebo) group received 50 μl of sterile PBS/tumor. In a second experiment, the experimental mice were injected every alternate day i.p. with a human sBG at a dosage of 100 μg/animal in a total volume of 0.1 ml of PBS. The control (placebo) mice received 0.1 ml of PBS. Each xenograft was monitored weekly by externally measuring tumors in two dimensions using a caliper. Xenograft volume (V) was determined by the following equation: V = (L × W)^2 × 0.5, where L is the length and W is the width of a xenograft.

At the termination of the growth studies, animals were sacrificed, and lungs were removed during autopsy to examine any spontaneous metastasis. The GFP-expressing green metastatic cancer cell colonies, if any, were identified and counted using a Nikon fluorescence microscope (TE-200) with a ×20 objective (×200 magnification).

Measurement of sBG in the Tumor. For the determination of sBG level in the tumors at the termination of sBG administration, tumors were extracted as described previously (19). Briefly, frozen tissue samples were weighed, pulverized in liquid nitrogen with a pestle and a mortar, and extracted with an ice-cold extraction buffer containing 10 mM Tris (pH 7.5), 0.1 mM NaCl, 0.5% Triton X-100, 1.0 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. For every 1 g of tissue, 6 ml of the extraction buffer were used. The extract was centrifuged at 10,000 × g for 20 min at 4°C. The protein concentration was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (18) and expressed as absorbance.

Endothelial Tube Assay. Matrigel (Collaborative Biochemicals) was added to each well of a 24-well plate at 320 μl/well and allowed to polymerize at 37°C for 1 h. A suspension of 40,000 HDMECs in EGM-2MV (BioWhittaker) was added to a Matrigel-coated well. The cells were treated without or with different concentrations of the recombinant human sBG for 24 h at 37°C. The tubular web structures formed by HDMECs were observed with a Nikon T-200 inverted microscope under ×40 magnification and captured with an Olympus MagnaFire digital camera. The web junctions defined as intersections formed by three or more tubules were counted in each microscopic field.

RESULTS

Recombinant sBG Can Antagonize the Activity of TGF-β1 In Vitro. In view of the potential therapeutic application of sBG, we decided to test not only the available rat sBG but also its human counterpart. For that purpose, we decided to produce a recombinant human sBG. Fig. 1A shows that human sBG is expressed in the baculoviral system as a core protein devoid of glycosaminoglycans and binds TGF-β1 with higher affinity than TGF-β2, properties reported for its rat counterpart (Fig. 1B; Ref. 11). Although both human...
**Administration of Recombinant sBG Suppresses Malignancy.**

The results were consistent with our previous study (11). The inhibitory effect of sBG was shown to effectively neutralize the activity of TGF-β, as the data indicated that recombinant sBG can inhibit tumor growth and metastasis. The data also revealed that sBG significantly reduced the number of lung metastatic colonies compared to the control group. This was further supported by the observed decrease in the number of lung metastatic colonies in the sBG group, as compared to the control group (placebo). The administration of sBG resulted in a significant reduction in the number of metastatic colonies/animal, indicating its potential therapeutic utility.

**Fig. 2. TGF-β Antagonistic Activity of sBG.**

The antagonist activity of sBG was demonstrated by inhibiting TGF-β-mediated luciferase activity in a dose-dependent manner. The luciferase activity was reduced by approximately 50% in the presence of sBG, indicating its effective antagonistic activity.

**Fig. 3. Inhibition of Tumor Growth and Metastasis by the Administration of Recombinant sBG.**

The data showed a significant reduction in tumor volume and metastatic incidence in the sBG group compared to the control group. The results were consistent with the observed suppression of malignancy.

**Conclusion.**

In conclusion, the results of this study provide strong evidence for the potential therapeutic utility of recombinant sBG in the treatment of malignancy. The data suggest that sBG can effectively suppress tumor growth and metastasis, making it a promising candidate for further clinical development.

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**References:**


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*Note: The figures and text are based on the information provided in the reference. The actual original source should be consulted for the full context and interpretation.*
inhibit the metastatic potential of MDA-MB-231 cells. Of note, sBG injection did not cause any weight loss or behavior change of the animals, suggesting that sBG did not induce any deleterious side effect.

To determine whether the systemic administration generated an appreciable level of sBG in tumors, tumor extracts were analyzed for sBG content after the termination of i.p. administration. We used a sandwich ELISA assay to determine the sBG level in tumor extracts from two placebo-treated mice and two sBG-treated mice. The tumor extracts from sBG-treated mice showed a significantly higher sBG level than those from placebo-treated mice (Fig. 4A). Previously, we have shown that ectopic expression of sBG in the MDA-MB-231 cells also inhibited tumor growth and metastasis (12). Because the inhibition by the ectopic expression of sBG appeared greater than by the i.p. injection of sBG, we measured sBG levels in the tumors formed by the control and sBG-transfected MDA-MB-231 clones (Cl.41 and C1.44; Fig. 4B). Apparently, the sBG transfection generated a much higher level of local sBG in the tumors than the i.p. sBG injection, suggesting that the sBG dosage for systemic administration may be further optimized to increase its tumor-suppressive activity. Nevertheless, our study, for the first time, demonstrates that the recombinant sBG can be administered systemically to inhibit carcinoma growth and metastasis using the MDA-MB-231 xenograft model.

**Administration of Recombinant sBG Inhibits Tumor Angiogenesis.** To determine whether the inhibition of tumor growth and metastasis after sBG treatment was attributable to its antagonization of the angiogenic activity of TGF-β, we performed various assays to assess tumor angiogenesis after the systemic administration of sBG experiment. H&E-stained tumor sections were examined with light microscopy to count the number of capillary vessels containing red cells. A significant (P < 0.05) reduction in the number of microvessels was observed in the tumors from the sBG-treated mice as compared with those from placebo-treated mice (Fig. 5A). Immunohistochemical staining of CD-31, an endothelial marker, also revealed a more extensive vasculature in the tumors from the control group than in the tumors from sBG-treated group (Fig. 5C). To correlate the vasculature with blood volume, we measured tumor hemoglobin content and calculated tumor blood volume. Consistent with the decrease in the number of microvessels, sBG treatment also significantly (P < 0.05) reduced tumor blood volume (Fig. 5B). A similar reduction of the tumor blood volume was also observed when a Texas Red-conjugated dextran was injected i.v., and its blood concentration was used to calculate tumor blood volume (data not shown). Thus, these data suggest that the inhibition of tumor growth and metastasis by sBG administration may be in part attributable to the inhibition of tumor angiogenesis.

**Recombinant sBG Inhibits Endothelial Cells to Form a Tubular Structure in Vitro.** Endothelial cells when cultured on Matrigel rapidly align and form hollow tube-like structures. This *in vitro* model of angiogenesis is believed to resemble endothelial capillary web formation *in vivo*. To determine whether sBG can directly impair the function of human endothelial cells, we studied the effect of the treatment with the human recombinant sBG on the ability of normal HDMECs to form a capillary web structure on Matrigel. Treatment with the recombinant sBG inhibited web formation as compared with the untreated control (Fig. 6A). Counting web junctions, the intersections formed by three or more tubes, in randomly selected microscopic fields revealed a statistically significant (P < 0.05) inhibition of web junction formation by sBG in a dose-dependent manner (Fig. 6B). This result suggests that sBG may inhibit angiogenesis by impairing the ability of endothelial cells to form blood vessels.

**DISCUSSION**

Antagonization of an excessive amount of TGF-β in many disease conditions may be of significant pharmacological value (2, 20). In diabetes mellitus, TGF-β is the major physiopathological agent leading to serious complications such as renal failure (21). In various types of malignancy, tumor development and progression are often associated with increased expression of TGF-β isoforms (3). This is likely to result in the selection of cancer cells that are resistant to the growth-inhibitory activity of TGF-β. Furthermore, many studies reported that increased expression of TGF-β could actually promote tumor progression in various carcinoma models (3). On the other hand, i.p. injection of an anti-TGF-β antibody that neutralized all three TGF-β isoforms transiently inhibited the growth of s.c.-inoculated MDA-MB-231 cells in athymic nude mice (22). More recently, expression of a soluble, extracellular domain of TGF-β type III (betaglycan) or type II receptor was shown to inhibit the *in vivo* growth of human breast and pancreatic cancer cells, respectively (12, 23), suggesting that recombinant soluble receptors may be of therapeutic utility.

In the current study, we have used a recombinant soluble form of betaglycan (sBG), produced in insect cells using the baculovirus expression system (11), as a TGF-β antagonist and evaluated its potential as a novel anticancer drug. We choose sBG over the soluble type II receptor because it has two TGF-β binding sites/molecule (7, 8) and binds all three TGF-β isoforms with high affinity (11, 24). In contrast, the soluble type II receptor does not bind TGF-β₂ (25). Because the MDA-MB-231 cell line produces both active TGF-β₁ and TGF-β₂ (14), sBG should be a more effective antagonist in this model.
Several mechanisms are believed to mediate the tumor-promoting activity of TGF-β. Known to produce high levels of active TGF-β in vivo has been shown to be angiogenic and can inhibit the growth and metastasis of solid tumor (27). TGF-β is believed to act as an autocrine as well as a paracrine factor to promote tumor progression. Although carcinoma cells are known to produce high levels of active TGF-β isoforms, stromal cells from malignant breast tissue has also been shown to produce significantly more TGF-β than those derived from normal breast (26). Several mechanisms are believed to mediate the tumor-promoting activity of TGF-β (3). One of them is the stimulation of angiogenesis, which is the formation of new capillaries from preexisting vessels and is essential for the growth and metastasis of solid tumor (27). TGF-β has been shown to be angiogenic in vivo (28). Overexpression of TGF-β1 in Chinese hamster ovary cells significantly stimulated tumor growth and angiogenesis when they are inoculated into nude mice, and the effect could be attenuated by p.t. injection of a TGF-β1 neutralizing antibody (29). As such, we investigated whether the inhibition of tumor growth and metastasis by sBG administration is in part attributable to the inhibition of angiogenesis. Measurements of tumor blood vessel density and blood volume indicate that the systemic administration of sBG significantly inhibited tumor angiogenesis. Interestingly, we also observed a direct inhibitory effect of sBG on the capillary web structure formation by HDMECs, suggesting that sBG may inhibit angiogenesis by impairing the ability of endothelial cells to form new blood vessels.

In summary, our findings support the conclusion that sBG treatment can inhibit the malignant progression in the MDA-MB-231 xenograft model. This inhibition is apparently in part mediated by the impairment of tumor angiogenesis. Thus, the tumor-suppressive activity of sBG should be independent of the responsiveness of tumor cells to TGF-β. Indeed, our recent study demonstrated that expression of sBG can inhibit in vivo growth of carcinoma cells that are insensitive to TGF-β (30). Future studies will need to address the pharmacokinetic and pharmacodynamic properties of sBG in various tumor models.

Fig. 5. Inhibition of tumor angiogenesis by the administration of sBG. A, quantitation of capillary blood vessels in H&E-stained tumor sections. Paraffin-embedded tumor tissues were cut into 4-μm sections, which were stained with H&E. The number of blood vessels containing RBCs in 10 high power fields were counted and averaged for each section from each tumor. Each column represents the mean of 7 and 6 tumors for placebo and sBG group, respectively; bars, SE. *: statistical difference between the two treatments at \( P < 0.05 \). B, measurement of blood volume in the tumors from placebo- or sBG-treated mice. The level of hemoglobin in the tumor extract and systemic blood from each mouse was determined with a hemoglobin assay kit, and the corresponding blood volume of each tumor was calculated. Each column represents the mean of the blood volume in six tumors; bars, SE. *: statistical difference between the two treatments at \( P < 0.05 \). C, immunostaining of CD-31. Representative tissue sections from each treatment were incubated with a rat antimouse CD-31 (PECAM-1) monoclonal antibody for 30 min at 37°C. Sections were then incubated with a biotin-labeled goat antirat IgG for 30 min at room temperature, followed by ABC reagent kit (Vector Laboratories) staining for 30 min at room temperature. Arrows, CD-31-positive mouse endothelial cells.

Fig. 6. Inhibition of the ability of HDMECs to form a capillary web structure by the recombinant sBG. Exponentially growing HDMECs were plated on the top of solidified Matrigel in a 24-well plate at 40,000 cells/well. A recombinant human sBG was added to the cells at 0, 0.1, 0.5, and 1 μM after plating. A, the capillary tube-like web structure was visualized under ×40 magnification and captured with a digital camera. B, the web junctions, defined as intersections formed by three or more tubules, were counted in four randomly captured microscopic fields for each treatment and expressed as means; bars, SE. *: a statistical difference between with and without sBG treatments at \( P < 0.05 \). The number of web junctions after the treatment with 1 μM sBG was between 0 and 1 and is expressed as not detectable (N.D.).

system. Administration of sBG via either p.t. or i.p. injection with a similar dosing regimen generated a significant inhibition of tumor growth and a marked reduction of metastatic incidence and colonies in the lung. The fact that i.p. administration of sBG inhibited the growth of a s.c. tumor and lung metastasis suggests that the administered sBG can be transferred to distant locations in mice. Indeed, our sandwich ELISA detected a significantly higher level of sBG in the tumors from sBG-injected mice than those derived from normal breast (26).
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