Antitumor Activity of a Recombinant Soluble Betaglycan in Human Breast Cancer Xenograft

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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. Bandyopadhyay 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 microvessel density detected with histological analyses and CD-31 immunostainings, 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.

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3 The abbreviations used are: TGF, transforming growth factor; sBG, soluble betaglycan; GFP, green fluorescent protein; HDMEC, human dermal microvascular endothelial cell; PAI-1, plasminogen activator inhibitor-1; p.t., peritumoral.

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 MDAMB-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, pE GFP-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 region) 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 125I-labeled TGF-β1 (in the absence or presence of the indicated concentrations of nonradioactive TGF-β) 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 Triis-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 125I-labeled TGF-β-complexed sBG was revealed by scanning in a PhosphorImager (Molecular Dynamics, Inc.). Quantitative densitometry of radiolabeled sBG was carried out.

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out using the ImageQuant software; data were analyzed using the Prism software.

Bioassays of TGF-β 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 × 10⁶ 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 × W²)/2, 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 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 15 min. sBG concentrations in the supernatants were determined by a two-antibody sandwich ELISA assay developed in our laboratory. An antihuman sBG polyclonal antibody was raised from rabbits by injecting the purified recombinant human sBG following standard immunization protocols (16). The antibody was precoated (1:5000 dilution) to the bottom of a 96-well immunoplate (Dynatech Laboratories, Inc.) as the capture antibody. The wells were washed with PBS containing 0.5% Tween 20 and blocked with 1% BSA. The purified human sBG and tumor extracts were added into the wells and incubated for 2 h at 37°C. The bound sBG was detected after subsequent incubation with an antihuman sBG goat antibody (R & D systems: 1:500 dilution), a polyclonal antigen IgG conjugated with horseradish peroxidase (Pierce; 1:30,000 dilution), and a horseradish peroxidase substrate mixture containing H₂O₂ and o-phenylenediamine dihydrochloride. The final reaction was stopped with 6 N H₂SO₄, and the absorbance was determined with a microplate reader at 492 nm. The sBG concentration in the tumor extract was determined from a standard curve using known amounts of human-sBG.

Assays for Tumor Vascularity. To determine the effect of sBG treatment on tumor angiogenesis, we measured the vascularity of excised tumors at the termination of sBG treatment. Tumor tissues were fixed in 10% neutral buffered formalin (Fisher Scientific) overnight at 4°C and embedded in paraffin. Sections of 4 μm were cut from the embedded tissue and stained with H&E. Sections were examined by light microscopy under ×400 magnification, and the number of blood vessels containing red cells from 10 high power fields was counted and averaged. CD31 immunostaining for mouse blood vessels was performed by incubating tumor sections with a rat antimonoclonal antibody (PharMingen) at 5 μg/ml for 30 min at 37°C. Sections were then incubated with a biotin-labeled goat antirat IgG (Zymed; 1:200 dilution) for 30 min at room temperature, followed by ABC reagent kit (Vector Laboratories) for 30 min at room temperature. Color reaction was performed with 3,3′-diaminobenzidine (Vector Laboratories) and counterstained with hematoxylin. All sections were coded and observed by a pathologist who was blinded for the study protocol.

Hemoglobin Assay. Hemoglobin levels in tumors were measured to corroborate the blood vessel density. Excised tumors were carefully cleaned for any external blood on their surface and then extracted as described above. Hemoglobin content in tumor extracts was measured using a hemoglobin assay kit (Sigma) following the manufacturer’s instruction. The hemoglobin content in systemic blood obtained through cardiac puncture at the termination of the experiment was also measured to obtain the blood volume/unit weight of tumor.

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-β 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 TGF-β isoforms were subjected to affinity labeling in solution with 100 pmol of ¹²⁵I-labeled TGF-β₂, the indicated concentrations of competing unlabeled TGF-β₁ or TGF-β₂, radioactive images were revealed with a PhosphorImager. The percentage of sBG-bound ¹²⁵I-labeled TGF-β₂ was estimated from densitometric analysis of the images using the ImageQuant Software. It is plotted against the TGF-β₁ concentration next to each image.

Fig. 1. The TGF-β₁ isomer-specific competition of the TGF-β₁ binding of baculoviral sBG. Baculoviral human (A) or rat (B) sBG was subjected to affinity labeling in solution with 100 pmol of ¹²⁵I-labeled TGF-β₂ and the indicated concentrations of competing unlabeled TGF-β₁ or TGF-β₂. Reactive images were revealed with a PhosphorImager. The percentage of sBG-bound ¹²⁵I-labeled TGF-β₂ was estimated from densitometric analysis of the images using the ImageQuant Software. It is plotted against the TGF-β₁ concentration next to each image.
Recombinant Soluble Betaglycan Suppresses Malignancy

Administration of Recombinant sBG Inhibits Tumor Growth and Metastasis. To investigate the potential therapeutic utility of sBG, we administered the recombinant sBG into human breast cancer xenograft-bearing nude mice. The MDA-MB-231/GFP cells were inoculated s.c. in both inguinal mammary fat pads of nude mice. In the first study, we tested whether local injection of sBG around the tumor could inhibit the tumor growth. When the growing tumors reached ~4 mm in diameter, we started injecting a rat recombinant sBG next to each tumor at a dose of 50 μg/tumor. The injection of sBG twice per week resulted in a significant (P < 0.05) reduction of the mean tumor growth rate when compared with the placebo injection over a period of 3 weeks (Fig. 3A). This result prompted us to test whether systemic administration such as i.p. injection of sBG would yield similar results. Therefore, in a second experiment, when the average tumor diameter reached ~4 mm, a recombinant human sBG was injected i.p. every alternate day at a dose of 100 μg/animal for 28 days. Similar to the p.t. injection, i.p. injection of sBG also generated a significant inhibition of tumor growth (Fig. 3B).

Because the tumor cells used in this study were labeled with the enhanced GFP, we were able to detect and count green micrometastatic colonies in the whole lung under an inverted fluorescence microscope. Both p.t. and i.p. administration reduced lung metastatic incidence, the percentage of the animal having lung metastasis, by ~60% (Fig. 3C). The average number of lung metastatic colonies/mouse were also reduced after p.t. (Fig. 3D) and i.p. (Fig. 3E) administration of sBG. These data indicate that recombinant sBG can

Fig. 3. Inhibition of tumor growth and metastasis by the administration of recombinant sBG. Exponentially growing MDA-MB-231 cells (3 x 10⁶) expressing the enhanced GFP were s.c. inoculated in both sides of the inguinal mammary fat pad of female athymic nude mice 4 weeks of age. When growing tumors reached an average diameter of >4 mm after ~5 weeks, animals were ranked according to their tumor volume and divided into two groups. A, both tumors in each mouse of the experimental group (n = 7) were injected twice a week peritumorally (next to the tumor) with a recombinant rat sBG at a dose of 50 μg/tumor in a total volume of 0.05 ml of PBS. Both tumors in each mouse of the control (placebo) group (n = 6) received 50 μl of PBS. Xenografts were measured externally in two dimensions using a caliper. Each point is the mean of 14 tumors for the sBG group and 12 tumors for the placebo group. Bars, SE. B, the experimental animals (n = 5) were injected every other day i.p. with a human recombinant sBG at a dose of 100 μg/animal in a total volume of 0.1 ml of PBS. The control (Placebo) group (n = 5) received an equal volume of PBS. Each point is the mean of 10 tumors; bars, SE. C, statistical difference between the sBG and placebo treatments at P < 0.05. C, at the termination of the tumor growth studies, animals were euthanized, and both lungs were removed during autopsy to examine any disseminated tumor cell metastasis. The GFP-expressing green metastatic cancer cell colonies, if any, were identified and counted using an inverted Nikon fluorescence microscope (TE-2000) with a ×20 objective lens (×200 magnification). Lung metastatic incidence is presented as the percentage of animals having lung metastatic colonies after p.t. or i.p. injection with PBS (Placebo) or sBG. The number of lung metastatic colonies in each animal observed with the microscope are presented in D after p.t. injection and in E after i.p. injection. The horizontal bars in both panels represent the average number of the metastatic colonies/animal.
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 Cl.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 tubules, 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 factor to promote tumor progression. Although carcinoma cells are known to produce angiogenic factors in vivo, the ability to their angiogenic potential can also be important to determine whether the administration of sBG can inhibit the malignant progression in the MDA-MB-231 xenograft model. This inhibition is apparent 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.

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.

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