A Novel Disintegrin Salmosin Inhibits Tumor Angiogenesis

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ABSTRACT

Salmosin is a snake venom-derived novel disintegrin that antagonizes platelet aggregation. In this study, we investigated its functional specificity in tumor angiogenesis. Salmosin significantly inhibited bovine capillary endothelial cell proliferation induced by basic fibroblast growth factor but had no effect on normal growth of the cell. The basic fibroblast growth factor–induced in vivo angiogenesis in the chorioallantoic membrane was disrupted by salmosin treatment without affecting normal embryonic angiogenesis. Adhesion of the bovine capillary endothelial cells to vitronectin was also inhibited by the binding of salmosin to the αvβ3 integrin. Both the metastatic-tumor growth and the solid-tumor growth that developed in mice were effectively suppressed by salmosin treatment. Several lines of experimental evidence strongly suggest that the tumor-specific antiangiogenic activity of salmosin disrupts tumor growth by blocking the αvβ3 integrin that is expressed on the vascular endothelial cell surface.

INTRODUCTION

Angiogenesis is the process of the formation of new blood vessels from preexisting blood vessels (1, 2). This process plays a key role in development, wound healing, and inflammation. Angiogenesis is also essential for the progression of solid tumors and is regulated in part by vascular cell adhesion molecules in smooth muscle and endothelial cells (3). The change in the angiogenic phenotype in tumors may be due to a change of balance between the positive and negative modulators involved in neovascularization (4–6). In a recent report, two cytokine-dependent pathways of angiogenesis were shown to exist and were defined by distinct vascular cell integrins, αvβ3 and αvβ5, that become expressed in angiogenic vascular cells, in which they played a critical role in angiogenesis induced by bFGF3 tumor necrosis factor α, vascular endothelial growth factor, and fragments of human tumors (7). Activation of the αvβ3 integrin stimulates the survival signal that facilitates blood vessel growth and differentiation, which indicates that signaling events by both cytokines and integrin receptors are closely associated with the growth of new blood vessels (8). Several endogenous angiogenic inhibitors have been identified: (a) IFN-α and -γ (9, 10); (b) IFN-inducible protein 10 (11, 12); (c) angiotatin and endostatin that specifically suppress endothelial cell proliferation (5, 13); (d) gro-β (14); (e) the 16-kDa NH2-terminal fragment of prolactin (15); and (f) platelet factor 4 (16, 17).

Disintegrins are a family of small proteins mainly derived from snake venoms (18). Most of the disintegrins contain the RGD or the Lys-Gly-Asp sequence, which is the structural motif recognized by the platelet fibrinogen receptor α2bβ3, and also act as potent antagonists of several integrins including αvβ1 and αvβ3 (19). There are several reports demonstrating that disintegrins containing the RGD sequence inhibit tumor metastasis by blocking tumor cell adhesion to ECMs (20, 21). Integrin αvβ3 was identified as a marker of angiogenic blood vessels in chick and human embryos (22). A monoclonal antibody against αvβ3 was able to inhibit angiogenesis by inducing apoptosis in the endothelial cells of the newly formed blood vessels (7, 8). The application of synthetic peptides containing the RGD sequence that inhibit ligand binding to integrin αvβ3 suppressed tumor-induced angiogenesis on the chick CAM (8). These findings suggest that disintegrins, synthetic RGD peptides, and anti-αvβ3 monoclonal antibodies may have the potential to be developed as anticancer agents. We have found and characterized the novel disintegrin salmosin derived from Korean snake venom (23). In this communication, we demonstrate that salmosin, a disintegrin containing the RGD sequence, inhibits tumor growth and bFGF-induced angiogenesis without affecting the proliferation of normal endothelial cells.

MATERIALS AND METHODS

Materials. Lewis lung carcinoma cells were purchased from American Type Culture Collection. C57BL/6 mice were from Charles River (Yokohama, Japan). The primary culture of BCE cells was obtained from bovine adrenal glands as described previously (24). ΔMPA expression vector was kindly provided by Dr. Yungdae Yun at Mogam Biotechnology Research Institute (Yong-In, Korea). Angiotatin was from Technoclone (Wien, Austria). Anti-αvβ3 monoclonal antibody was from Serotec (Oxford, United Kingdom). The Resource S column was purchased from Pharmacia (Uppsala, Sweden). Protein standards for electrophoresis and SDS-PAGE gel were from NOVEX (San Diego, CA). All of the other reagents were of the highest purity available from commercial sources.

Purification of Recombinant Salmosin. The cDNA of salmosin was cloned as a fused gene into the ΔMPA vector, and the cloned vector was transformed into Escherichia coli MC1061. The transformant was grown in Luria-Bertani medium at 37°C to reach the absorbance of 0.5 at 600 nm. Arabinose (1%, w/v) was then added and followed by further incubation for 16 h. The cells were harvested and resuspended in 20 mM Tris-HCl (pH 8.0). They were completely lysed by a microfluidizer (Microfluidics), and the inclusion body was collected by centrifugation. The pellet was washed several times with 20 mM Tris-HCl (pH 8.0) containing 0.1% Triton X-100 to remove contaminants, and the inclusion body was recovered. An effective refolding procedure was carried out to obtain the active salmosin fusion protein. Re-folded fusion protein was proteolytically cleaved and applied directly to Resource S (Pharmacia) cation exchange column. The recombinant salmosin was eluted with 0.2 M NaCl and then dialyzed against PBS.

BCE Cell Proliferation Assay. The cells were maintained in DMEM containing 3 ng/ml bFGF with 10% FCS. The proliferation assay was performed as described previously (5). BCE cells were plated onto gelatinized 24-well culture plates and incubated at 37°C, 5% CO2 for 24 h. Immediately after the incubation, the cells were treated with the sample by replacing the media with 0.25 ml of DMEM containing 5% FCS. After a 20-min incubation, cells were treated with bFGF (1 ng/ml) dissolved in the media. After an additional 72-h incubation, cells were dispersed in trypsin and counted.

Chick CAM Assay. Three-day-old fertilized eggs were carefully cracked and sealed with transparent tape. After 3 days of incubation in 60% humidity at 37°C, a methylcellulose disc containing 50 μg of salmosin or 50 μg of...
angiostatin was implanted on the CAM of individual embryo. After a 48-h incubation, embryos and CAMs were observed under stereomicroscope.

BFGF-induced Angiogenesis Assay. Angiogenesis was induced in the chick embryo by injecting bFGF (6 ng/embryo) on the CAM of 10-day-old embryos. After 24 h, salmosin (5 mg) or anti-\( \alpha_v\beta_3 \) monoclonal antibody (5 mg) was applied to the CAM. Three days later, the blood vessels were observed by dissecting microscope.

BCE Cell Adhesion Assay. BCE cells were dissociated by treatment with trypsin-EDTA, washed three times in PBS, and resuspended in serum-free DMEM. Before the addition of the cells to each well, the cells (5 \( \times \) 10^5) were preincubated with salmosin, anti-\( \alpha_v\beta_3 \) monoclonal antibody, synthetic RGD peptide (GRGDSP), or synthetic Arg-Gly-Glu peptide (GRGETP) for 20 min at 37°C. After the incubation, the cells were added to each well and incubated for 1 h at 37°C in 5% CO\(_2/95\% \) air. Unattached cells were removed by washing with PBS. Attached cells were fixed and stained with Coomassie Blue. Absorbance at 540 nm of the individual well was measured to determine the relative number of cells.

Growth Inhibition of Pulmonary Metastatic Tumor. Lewis lung carcinoma cells (1.5 \( \times \) 10^5) were injected into the lateral tail veins of 8-week-old male C57BL/6 mice. Four days later, recombinant salmosin (1.25 mg/kg/day) was administered i.v. to the mice once daily. Four weeks later, the mice were killed, and the lungs were removed. The number of lung tumor colonies were counted by dissecting microscope (25). The lungs were used for histochemical analysis.

Immunohistochemical Analysis. Lung tissue was fixed in 4% formaldehyde and embedded in paraffin according to the standard procedure. Sections (4 \( \mu \)m thick) were permeabilized with trypsin at 37°C for 10 min and washed in PBS. These sections were stained with rabbit antiserum against human factor VIII (DAKO PAP kit), and the antibody binding was detected by sequential incubation of the sections with swine antirabbit serum. Color staining was performed by substrate reaction with 3-amino-9-ethylcarbazole (AEC). The sections were counterstained with Mayer’s hematoxylin. Histological analysis of paraffin sections was performed with H&E.

Growth Inhibition of s.c. Solid Tumor. Lewis lung carcinomas cells (1 \( \times \) 10^6) were injected s.c. into dorsal midline of C57BL/6 mice (5). When

![Fig. 1. SDS-PAGE of natural and recombinant salmosin. Natural salmosin (Lane 1) isolated from the snake (Agkistrodon halys brevicaudus) venom and purified recombinant salmosin (Lane 3) were analyzed by SDS-PAGE using 4–20% polyacrylamide gradient gel. Molecular mass markers are shown in Lane 2.](cancerres.aacrjournals.org)
tumors increased to 100–200 mm³ in volume, the mice were randomized into two groups. One group received salmosin (10 mg/kg mouse) in PBS via s.c. injection once daily at a site distant from the tumor. The other group received comparable injections of PBS alone. The experiments were terminated when the control mice began to die.

RESULTS

Purification of Recombinant Salmosin. The cDNA-encoding salmosin was cloned and expressed in E. coli as a form of fusion protein. The expressed fusion protein was refolded and then cleaved at a specific site to remove the fusion partner. In the final purification step, the recombinant salmosin was recovered by fast protein liquid chromatography fractionation. The purified recombinant salmosin migrated as a single band on SDS-PAGE (Fig. 1). When we analyzed the purity of salmosin by high-performance liquid chromatography gel filtration and reverse-phase high-performance liquid chromatography, no contamination was detected; and its molecular identity with the native protein that was isolated from the snake (Agkistrodon halys brevicaudus) venom was demonstrated by mass spectrometric analysis and by NH₂-terminal amino acid sequencing.

Inhibition of BCE Cell Proliferation by Salmosin. To examine the ability of salmosin to inhibit angiogenesis, we used a BCE cell proliferation assay system that was developed for the study of tumor angiogenesis (5). Salmosin was able to inhibit the proliferation of BCE cells induced by bFGF in a dose-dependent manner. One-half-maximal inhibition of bFGF-induced BCE cell proliferation was observed with a salmosin concentration of 0.1–0.2 µg/ml corresponding to 13–27 nM (Fig. 2 A). The bFGF-induced BCE cells undergo remarkable morphological change into spherical shape by treatment with salmosin (Fig. 2, C and D). However, it is interesting to note that no inhibition of the cell proliferation was observed in the absence of bFGF when the cells were treated with 20 µg/ml salmosin, which is required for maximal inhibition of bFGF-induced proliferation (Fig. 2 A, insert). As shown in Fig. 2, the cell proliferation was highly stimulated over 2-fold by bFGF treatment. It is also noteworthy to observe that there was no visible change in the new blood vessel formation when salmosin (50 µg/embryo) was applied to 6-day-old embryos in an angiogenic CAM assay (Fig. 3). These findings strongly suggest that salmosin inhibits tumor-induced angiogenesis without affecting the preexisting blood vessels or the angiogenesis that is critical for normal physiological process. Further investigation revealed that the anti-αvβ3 monoclonal antibody significantly inhibits BCE cell proliferation induced by bFGF (Fig. 2 B). When these results are taken together, it is possible to postulate that salmosin binds to the αvβ3 integrin, which is associated with bFGF-induced BCE cell proliferation.

Inhibition of bFGF-induced in Vivo Angiogenesis by Salmosin. To assess the effect of salmosin on bFGF-induced angiogenesis as an in vivo model of tumor-derived neovascularization, we observed whether salmosin is capable of interrupting the angiogenesis by the disintegrin treatment in CAM. It was previously reported that integrin αvβ3 antagonists such as RGD peptide and anti-αvβ3 monoclonal antibody inhibited tumor-induced angiogenesis by disrupting the formation of new blood vessels (8). After bFGF induction for 24 h, CAMs treated with PBS were well vascularized (Fig. 4 A), whereas those treated with anti-αvβ3 monoclonal antibody (Fig. 4 B) or salmosin (Fig. 4 C) were not able to produce such a blood vessel formation. It was surprising to find that salmosin disrupted bFGF-induced in vivo angiogenesis by generating discontinuous blood vessels in 72 h, yet had no effect on preexisting vessels in the same CAMs (Fig. 4 C). These microscopic observations further strengthen the hypothesis that salmosin inhibits tumor angiogenesis by targeting the bFGF-induced αvβ3 integrin.

Inhibition of BCE Cell Adhesion by Salmosin. To confirm that the inhibition of BCE cell proliferation by salmosin is the consequence of direct-binding of salmosin to the αvβ3 integrin, which is a...
vitronectin receptor on the surface of BCE cells, we investigated whether salmosin is capable of inhibiting BCE cell adhesion to vitronectin coated onto 96-well plates. The adhesion of BCE cells to vitronectin was highly suppressed by preincubating the bFGF-stimu-

lated cells with salmosin (Fig. 5A). Similarly, the anti-αvβ3 monoclonal antibody strongly inhibited the cell adhesion to salmosin (Fig. 5B). Synthetic RGD peptide (GRGDSP) was also able to prevent the cells from adhesion to either vitronectin or salmosin (Fig. 5, A and B). These results clearly demonstrate that salmosin binds to the αvβ3 integrin on BCE cells and thereby blocks integrin-mediated cell adhesion.

Inhibition of Metastatic Tumor Growth by Salmosin. Disintegrins block lung tumor colonization through the inhibition of tumor cell attachment to endothelium (20, 21). However, thus far, there is no report demonstrating that disintegrins inhibit the growth of lung metastatic tumor. To assess whether salmosin affects metastatic tumor growth, metastatic colonies were developed in mice by injecting Lewis lung carcinoma cells into the tail vein. Salmosin markedly prevented the metastatic tumor growth at a dose of 1.25 mg/kg/day given i.v. (Table 1). To further investigate the consequences of salmosin treatment on the pulmonary metastatic tumor, histochemical analyses were carried out. In contrast to the clearly visualized metastatic tumor in the group of control mice, few tumor colonies were detected in the salmosin-treated animals (Fig. 6). Moreover, immunohistochemical analysis of the tumor sections revealed significant inhibition of tumor neovascularization in salmosin-treated animals (Fig. 7). This result can be explained by the antiangiogenic function of salmosin that strongly inhibits bFGF-induced BCE cell proliferation, presumably by blocking the αvβ3 integrin, which is essential for tumor angiogenesis. After the salmosin treatment, there was no recognizable evidence of toxicity in any of the mice tested. It is evident that the observed suppression of metastatic tumor growth is closely related to the angiogenic event that is necessary for secondary tumor growth.

Table 1 Growth inhibition of pulmonary metastatic Lewis lung carcinoma by salmosin treatment

<table>
<thead>
<tr>
<th>Salmosin (mg/kg mouse)</th>
<th>No. of mice</th>
<th>Average no. of lung tumor colonies</th>
<th>Inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>15 ± 6</td>
<td>0</td>
</tr>
<tr>
<td>1.25</td>
<td>4</td>
<td>0.8 ± 0.7</td>
<td>93</td>
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Lewis lung carcinoma cells (1.5 × 10⁶) were injected into the lateral tail veins of C57BL/6 mice. After 4 days, salmosin in PBS was injected into one group of mice once daily. A control group received PBS without salmosin. Four weeks after the injections, mice were killed, and lungs were removed. The number of lung tumor colonies in each mouse were counted by dissecting microscope.
Inhibition of Solid Tumor Growth by Salmosin. A further attempt was made to examine the *in vivo* role of salmosin in the growth of solid tumor in the mice. Lewis lung carcinoma cells were injected s.c. into the dorsal midline of mice and grown to a mass of at least 100 mm³. When salmosin was administered to the tumor-bearing mice via s.c. injection once daily at 10 mg/kg, the tumor growth was notably reduced in about a week relative to that in the group of control mice, which were treated with PBS (Fig. 8). The observed inhibitory effect of salmosin on the solid-tumor growth may be due to the antiangiogenic effect of salmosin.

Fig. 6. Histochemical analysis of pulmonary metastatic Lewis lung carcinoma. Lung tissues isolated from the PBS- or salmosin-treated mice were fixed for 4 h in Bouin’s solution before being embedded in paraffin. Sections were stained with H&E. *A*, ×40; *B*, ×200.

**Inhibition of Tumor Angiogenesis**

**Fig. 7.** Inhibition of tumor-induced angiogenesis in pulmonary Lewis lung carcinoma tissue by salmosin. Immunohistochemical analysis was performed as described under "Materials and Methods." Lung tumor tissues were cut and stained with polyclonal antibody against factor VIII. Positive color detection was carried out by substrate reaction with 3-amino-9-ethylcarbazole (AEC). *A* and *C*, PBS-treated control tumor sections; *B* and *D*, salmosin-treated tumor sections. Scale bar, 30 μm.
INHIBITION OF TUMOR ANGIOGENESIS

Fig. 8. Inhibition of solid-tumor growth of Lewis lung carcinoma by salmosin. Lewis lung carcinoma cells (1 × 10⁶) were s.c. implanted into the dorsal midlines of 20 mice. The animals were randomly divided into two groups when the tumor size increased to 100–200 mm³, after which the injection of salmosin (10 mg/kg/day) or of PBS was started.

DISCUSSION

Salmosin is a novel disintegrin containing the RGD sequence that was discovered and characterized previously in our laboratory (23). We have demonstrated in the present work that recombinant salmosin expressed in E. coli strongly inhibits bFGF-induced BCE cell proliferation and tumor growth. Because salmosin was initially identified as a potent α₃β₃ antagonist in platelet aggregation, we investigated the role of salmosin in integrin-mediated BCE cell proliferation and bFGF-induced in vivo CAM angiogenesis as model systems of neo-vascularization in tumor.

Cell survival and proliferation rely on signals induced by growth factors and adhesion proteins in the ECM (26–27). Integrin-mediated cell adhesion modulates cell survival and proliferation in vitro (26–30). However, multiple integrin species are associated with various adhesive proteins in vivo. Thus, how an individual matrix protein or integrin affects the cell survival correlated to physiologically relevant ECM remains unclear. We have shown here that the α₃β₃ integrin plays a critical role in BCE cell adhesion and proliferation. Several reports demonstrated that distinct β1 integrins can mediate the survival of mammalian cells and Chinese hamster ovary cells in vitro, whereas α₃β₃ mediates melanoma cell survival in collagen and the survival of proliferating vascular cells in vivo (8, 28, 30, 31). Integrin α₃β₃ recognizes several ECM proteins including vitronectin (32). There are several reports indicating that cellular adhesion and angiogenesis may occur as a combined process (33, 34). Neovascularure in tumor expresses the integrin α₃β₃, which was identified as one of molecular markers in the angiogenic vessels and which plays an important role in human breast tumor growth (35). It is widely accepted that tumor-induced angiogenesis is initiated by angiogenic cytokines such as bFGF and vascular endothelial growth factor that are expressed in the tumor itself. This process depends on vascular cell migration and invasion, which are regulated by cellular adhesion receptors (7). The dependence of angiogenesis on vascular cell adhesive events in vivo is evidenced by the fact that antagonists of α₃β₃ integrin block angiogenesis in chick CAM induced by bFGF and by fragments of human tumor (8). It was also demonstrated that the α₃β₃ integrin is expressed in blood vessels induced by bFGF in tumor but not in normal skin, and, thus, is identified as a specific angiogenic marker of tumor vascular tissue. Angiogenin is a well-known indirect angiogenic factor that supports endothelial cell adhesion and blood vessel formation. This angiogenic function is inhibited by the synthetic RGD peptide species (36). In the present study, we have shown that salmosin strongly inhibits bFGF-induced BCE cell proliferation and the cell adhesion to vitronectin by binding to α₃β₃ integrin. This inhibitory action of salmosin may lead to cell cycle arrest. However, it is surprising to find that salmosin has no effect on the normal physiological angiogenesis that is essential for embryonic development in CAM (Figs. 3 and 4). It was indicated in a recent report that triflavin (37) and accutin (38), which are members of a disintegrin family derived from snake venom, inhibited human umbilical vein endothelial cell adhesion/migration in vitro as well as in vivo angiogenesis in CAM. We also demonstrated that salmosin disrupts bFGF-induced CAM angiogenesis in the same manner that anti-α₃β₃ monoclonal antibody does, at least under microscopic observation (Fig. 4). Further investigation was carried out to define whether tumor growth is affected by salmosin that inhibits BCE cell proliferation. Several lines of experimental evidence clearly demonstrated that salmosin suppresses metastatic and solid tumor growth of Lewis lung carcinoma (Figs. 6–8). Therefore, based on the data presented here, it is possible to conclude that the suppression of tumor growth by salmosin is closely associated with the inhibition of neovascularization via blocking the α₃β₃ integrin.

ACKNOWLEDGMENTS

We thank Dr. Soo-Ik Chang (Chungbuk National University, Chung-Ju, Korea) for helpful discussion and Prof. Soung-So Kim (Yonsei University, Seoul, Korea) for the kind donation of BCE cells.

REFERENCES


4 Unpublished data.

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