Biochemical and Biophysical Research Communications **275,** 169–173 (2000)
doi:10.1006/bbrc.2000.3130, available online at http://www.idealibrary.com on IDEAL®

Suppressive Mechanism of Salmosin, a Novel Disintegrin in B16 Melanoma Cell Metastasis

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Received June 13, 2000

We have previously reported that salmosin, a novel disintegrin, was isolated from Korean snake (*Agkistrodon halys brevicaudus*) venom and significantly inhibited solid tumor growth in mice by perturbation of tumor-specific angiogenesis via blocking $\alpha v\beta 3$ integrin expressed on vascular endothelial cells. In this study, we investigated the functional specificity of salmosin in tumor cell metastasis. Recombinant salmosin expressed in *E. coli* that has the RGD sequence markedly inhibited both B16F10 melanoma cell adhesion to the extracellular matrix proteins as well as B16F10 melanoma cell invasion through Matrigel-coated filter. The inhibition by salmosin can be caused by blocking integrins expressed on the surface of B16F10 melanoma cells. Salmosin significantly inhibited the proliferation of B16F10 melanoma cells on the plate coated with collagen I in a dose-dependent manner. *In vivo* B16F10 melanoma experimental metastasis, salmosin showed remarkable significant inhibitory effect on lung tumor colonization in a concentration-dependent manner. These results clearly demonstrate that antimetastatic activity of salmosin resulted from blocking the integrin-mediated adherence and $\alpha v\beta 3$ integrin-mediated proliferation of the melanoma cells.

Key Words: **disintegrin; salmosin; tumor metastasis; snake venom.**

Tumor invasion and metastasis are the processes that lethally spread cancer cells throughout the body and closely related to the adhesive interaction between cells and extracellular matrix. In the process of tumor metastasis, tumor cells can cause endothelial cells to retract, exposing the subendothelial basement membrane and allowing the tumor cells efficiently to adhere to extracellular matrix (ECM) proteins of the surrounding stroma (2–4). These matrix proteins promote cell adhesion by binding to specific cell surface receptors, including a member of integrin family. Structurally, each integrin is a heterodimer consisting of a subunit noncovalently associated with α,β subunit. The $\beta 1$ subfamily has been considered to be the primary mediator of extracellular matrix adhesions. There is a report indicating that $\beta 1$ integrins may have other functions, such as direct mediation of cell-cell adhesion (5, 6). The $\beta 2$ subfamily that is found on leukocytes contains a receptor mediating cell-cell interactions. The $\beta 3$ subfamily includes the platelet glycoprotein IIb/IIIa complex and the vitronectin receptor. The $\beta 3$ subfamily may be important in the development of tumor invasiveness and malignancy (7). The integrin receptor complex that is spanned the plasma membrane links the integral cytoskeletal network of a cell with the extracellular environment. Common or characteristic core sequences in cell adhesion molecules such as fibrinogen, vitronectin and laminin have been found to contribute to cell adhesion and to the spread or integration of cell (8). The investigators suggested that tumor formation and metastasis are closely associated with the role of integrins (9–14). Overexpression of fibronectin receptor $\alpha 5\beta 1$ suppressed the transformed phenotype of Chinese hamster ovary cell (9). Integrin $\alpha 5\beta 1$ was reduced in the ras-transformed rodent cells (10). Superfibronectin that is a polymeric fibrillar form of fibronectin prevented tumor metastasis and tumor formation (15). Integrin $\alpha v\beta 3$ is a specific marker of the most malignant cells, i.e., (7), suggesting a role for this adhesion receptor in the malignant growth of human melanoma. Integrin αv gene expression and the resulting adhesive phenotypes are directly involved in the proliferation of human melanoma *in vivo* (16). In the recent work, we have found and char-

Abbreviations used: PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; ECM, extracellular matrix; RGD, arginyl-glycyl-aspartic acid.

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0006-291X/00 $35.00

acterized the novel disintegrin salmosin derived from Korean snake (*Agkistrodon halys brevicaudus*) venom (17) and demonstrated that salmon markedly inhibited tumor angiogenesis and solid tumor growth by perturbation of integrin $\alpha v\beta 3$ expressed in endothelium (18). Therefore, it is possible to propose that a novel disintegrin salmosin prevents tumor metastasis by blocking adherence mechanisms of tumor cells in the host.

In this paper, we demonstrate that salmosin strongly inhibits experimental tumor metastasis.

MATERIALS AND METHODS

Materials. B16F10 melanoma cells were obtained from Mogam Biotechnology Research Institute, Korea. C57BL/6 mice were from Charles River, Japan. ΔpMA expression vector was provided by Dr. Youngdae Yun at Mogam Biotechnology Research Institute, Korea. Resource S column was purchased from Pharmacia (Uppsala, Sweden). Protein standards for electrophoresis and SDS–PAGE gel were from NOVEX (San Diego, CA). All other reagents were of the highest purity available from commercial sources.

Purification of recombinant salmosin. Recombinant salmosin was purified as described previously (19). Briefly, the recombinant *E. coli.* cells that can express salmosin were completely lysed by microfluidizer (Microfluidics, U.S.A.), and the inclusion body was collected by centrifugation. The active salmosin fusion protein was obtained by an effective refolding process. Refolded fusion protein was proteolytically cleaved and applied directly to Resource S (Pharmacia, Sweden) cation exchange column. The recombinant salmosin was eluted with 0.2 M NaCl and then dialyzed against phosphate-buffered saline (PBS).

B16F10 melanoma cell adhesion assay. Various amounts of solubilized fibronectin, vitronectin, collagen I, collagen IV, and laminin in PBS were added to 96-well plates and incubated overnight at 4°C. The plates were washed and incubated for 1 h with 1% bovine serum albumin to block unbound surface. Prior to addition of the cells to each well, the cells (5×10^4) were preincubated with salmosin 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 with methanol and stained with crystal violet. Absorbance at 550 nm of the individual well was measured to determine the relative cell number.

B16 melanoma cell invasion assay. The chemoinvasion assay was carried out as described (20). Briefly, polycarbonate filters were coated with 50 μg of Matrigel and placed in a Boyden blind well chemotaxis chamber. Basic FGF solubilized in serum-free medium was placed in the lower compartment of the Boyden chamber. Cell suspensions (3×10^5) in RPMI 1640 medium without fetal bovine serum were loaded into the upper compartment of the Boyden chamber with salmosin (10, 20 μg/ml) being tested. After incubation for 5 h at 37°C in 5% CO_2, 95% air, the filters were fixed with methanol and stained with Giemsa. The cells invading through the basement membrane were counted.

B16F10 melanoma cell proliferation assay. The cells (8000 cells per well) were plated onto 24-well tissue culture plates coated with collagen I and were incubated in RPMI 1640 medium containing 5% fetal calf serum for 24 h, and each sample in triplicate was added to cells. After 72 h, adherent and non-adherent cells were dispersed in trypsin and counted.

Experimental metastasis assay. B16F10 melanoma cells were detached with 0.02% EDTA and resuspended gently to 7.5×10^5/ml in RPMI 1640 medium. Salmosin (250, 500, and 1250 μg/kg mouse) was then mixed with the cells in RPMI 1640 medium without serum. Single-cell suspension of 200 μl aliquots containing the indicated amount of salmosin or PBS were injected into the lateral tail veins of mice. Fourteen days later the mice were sacrificed and the number of lung melanoma colonies were counted by dissecting microscope. The lungs were fixed in Bouin's solution and used for histochemical analysis.

Histochemical analysis. Lung tissue was fixed for 4 h in Bouin's solution and embedded in paraffin according to standard procedure. Sections (4 μm thick) were permeabilized with trypsin at 37°C for 10 min and washed in PBS. These sections were stained with hematoxylin and eosin, and then mounted.

RESULTS

Inhibition of B16 melanoma cell adhesion to extracellular proteins and invasion through Matrigel matrix. Cell adhesion plays an important role in tumor cell metastasis and organ invasion when tumor cells attach to and degrade the basement membrane and subsequently migrate into the underlying stroma. To assess whether salmosin is capable of inhibiting tumor metastasis, both *in vitro* cell adhesion test and invasion assay were performed. In the *in vitro* cell adhesion assay, premixed B16F10 melanoma cells with salmosin or PBS were applied to 96-well plates coated with various ECM proteins. After 2 h, salmosin significantly inhibited adhesion of B16F10 melanoma cells to fibronectin, vitronectin, type I collagen and type IV collagen in a dose-dependent manner (Fig. 1A). Salmosin appeared to be the most effective in inhibiting adhesion of B16F10 melanoma cells to type I collagen and vitronectin. The half-maximal inhibitions of the cell adhesions were observed with salmosin concentration of approximately 100 nM in type I collagen and vitronectin. These results indicate that salmosin has strong binding to type I collagen receptor and vitronectin receptor compared to fibronectin, type IV collagen, and laminin receptor. It is noteworthy to observe that synthetic peptide GRGDS (500 μM) significantly inhibited the adhesion of B16F10 melanoma cells to fibronectin, but did not interfere with the B16F10 melanoma cell adhesion to laminin (Fig. 1B). Interestingly, however, salmosin inhibited the adhesion of cells to both proteins. The dissimilar inhibitory effect of salmosin containing RGD sequence and GRGDS peptide on the adhesion of the cells to laminin remains to be understood in molecular level. The inhibitory activity of GRGDS on the cell adhesion to the ECM proteins being tested except laminin was similar to salmosin. However, the inhibitory efficacy of salmosin was much higher than that of GRGDS. It is evident that salmosin inhibits B16F10 melanoma cell adhesion by blocking the membrane integrins presented on the surface of the cells. To examine the inhibition of B16F10 melanoma cell invasion through ECM by salmosin, *in vitro* Matrigel assay system was employed. Experimental observation revealed that Salmosin markedly reduced the ability of B16F10 cells to invade the Matrigel by 80–90% compared to serum free media-treated control

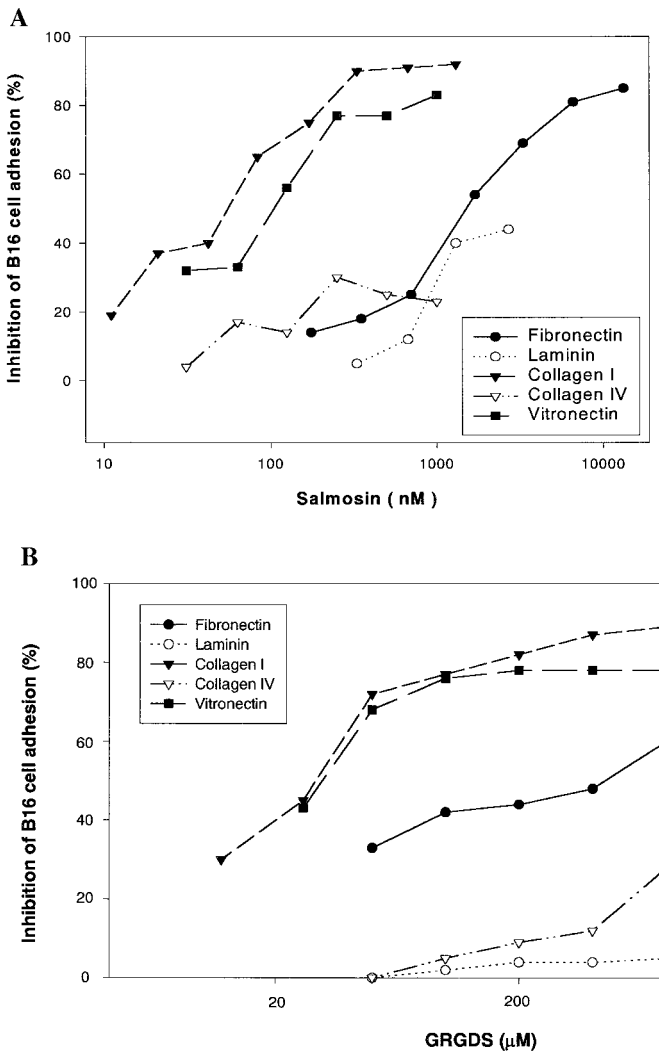

FIG. 1. Inhibition of B16 melanoma cell adhesion to ECM proteins by salmosin and synthetic peptide GRGDS. B16 melanoma cells were preincubated with salmosin (A) or synthetic peptide GRGDS (B) for 30 min prior to addition of the cells into fibronectin-, laminin-, collagen I-, collagen IV- and vitronectin-coated wells. The wells were rinsed after 1 h incubation and adhesion was determined colorimetrically. Each point represents the mean of triplicate.

B16F10 melanoma cells (Fig. 2). Taken together these results, it is possible to postulate that salmosin can reduce tumor cell invasiveness by suppressing integrin-mediated cell adhesion to ECM proteins in the basement membrane.

Inhibition of B16F10 melanoma cell proliferation. Integrin $\alpha v\beta 3$ play a critical role in melanoma cell survival in collagen gel. Integrin-mediated cell adhesion modulates cell survival and proliferation *in vitro.* To examine the ability of salmosin to inhibit the growth of B16F10 melanoma cells in collagen, we employed tumor cell proliferation assay system that was developed for the study of tumor metastasis. Salmosin was able to inhibit the proliferation of B16F10 on the

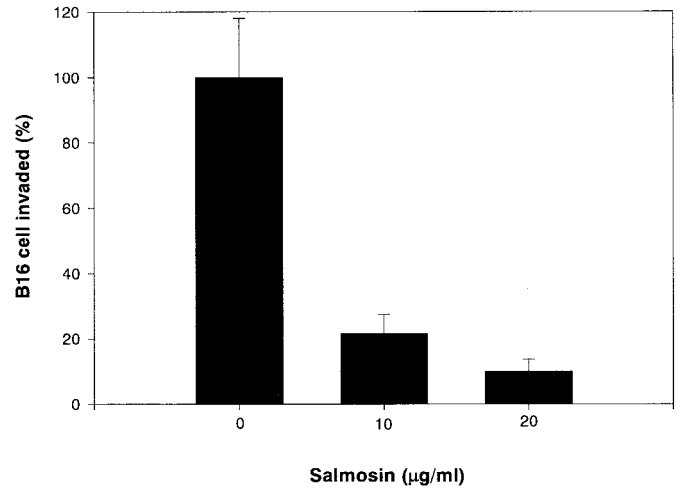

FIG. 2. Inhibition of B16 melanoma cell invasion *in vitro* by salmosin. Polycarbonate filters were coated with 50 μg of Matrigel and placed in a Boyden blind chemotaxis chamber. Cell suspensions (3×10^5) in RPMI media without fetal calf serum were loaded into the upper compartment of the Boyden chamber with salmosin being tested. The concentrations of salmosin were 0, 10, and 20 μg/ml. After incubation for 5 h at 37°C in 5% CO_2, the filters were fixed with methanol and stained with Giemsa. The cells invading through the Matrigel were counted. Each bar represents standard error of mean.

plate coated with type I collagen in a dose-dependent manner (Fig. 3). Half-maximal inhibition of the cell proliferation was observed with salmosin concentration of approximately 20 μg/ml corresponding to approximately 2.7 μM in both type I collagen. Further investigation revealed that anti-$\alpha v\beta 3$ monoclonal antibody or anti-$\beta 1$ monoclonal antibody significantly inhibited the cell proliferation (Fig. 3). These findings strongly suggest that salmosin is capable of suppress-

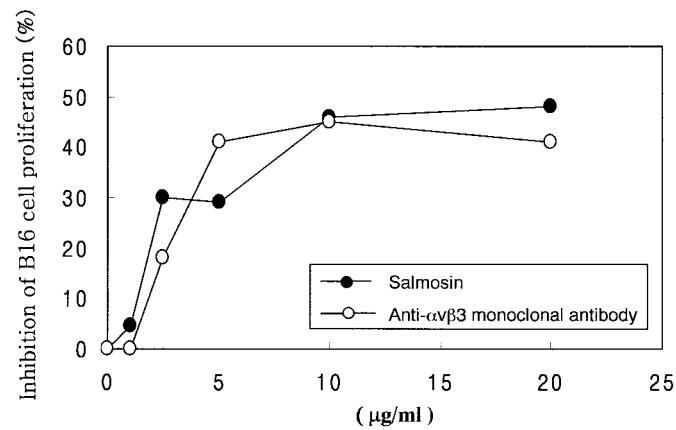

FIG. 3. Inhibition of B16 melanoma cell proliferation by salmosin. B16F10 melanoma cells were incubated with recombinant salmosin and anti-$\alpha v\beta 3$ monoclonal antibody on the 24-well culture plates coated with collagen type I for 72 h. The wells were rinsed and adherent cells were counted. Each point represents the mean of triplicate.

TABLE I

Inhibition of Experimental Metastasis by Salmosin

Salmosin (μg/kg mouse)	Number of mouse	Average number of lung tumor colony	Inhibition (%)
0	8	144 ± 40	0
250	7	49 ± 22	66
500	7	3 ± 2	98
1250	6	1 ± 1	99

ing B16F10 melanoma cell proliferation by blocking $\alpha v\beta 3$ or $\beta 1$ integrin.

Inhibition of experimental metastasis. To investigate the inhibitory effect of salmosin on tumor metastasis *in vivo*, salmosin was coinjected with B16F10 melanoma cells (5×10^5). It was surprising to observe the marked reduction of metastatic colonies compared to the PBS-treated control group in the lungs of C57BL/6 mice (Table 1 and Fig. 4). The inhibition of colonization caused by salmosin treatment was dose-dependent; lower dose (250 μg/kg mouse) of salmosin was able to inhibit the formation of the majority of the colonies in the lung, but few colonies were detectable when salmosin was administered with higher dose (Table 1 and Fig. 4). As demonstrated in Fig. 2, it is evident to visualize that salmosin greatly inhibits experimental metastasis in the mice. The inhibition of lung tumor colonization by salmosin was not due to cytotoxicity since incubation of the B16F10 melanoma cells *in vitro* with salmosin did not affect their subsequent proliferation rate. These experimental evidence strongly suggest that salmosin can inhibit tumor metastasis by acting as an antagonist of integrin receptors on the surface of tumor cells. The hematoxylin and eosin-stained sections of control mouse lung displayed the majority of metastases, but it was hard to detect the metastases in the lungs of salmosin-treated group even in the microscopic inspection (Fig. 5). Based on the results obtained in the present work, it is possible to conclude that the tremendous suppression of tumor metastasis elicited by salmosin is closely related to the inhibited B16F10 melanoma cell adhesion and invasion to ECM via blocking integrins.

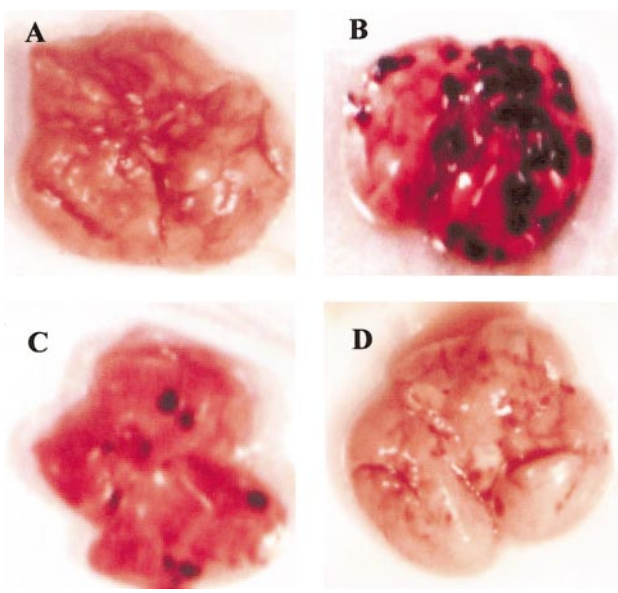

FIG. 4. Inhibition of experimental metastasis of B16 melanoma by salmosin. Lungs were isolated from mice developed for experimental metastasis. (A) Normal mouse lung. (B) PBS-treated mice. (C) 250 μg salmosin/kg mouse. (D) 1250 μg salmosin/kg mouse.

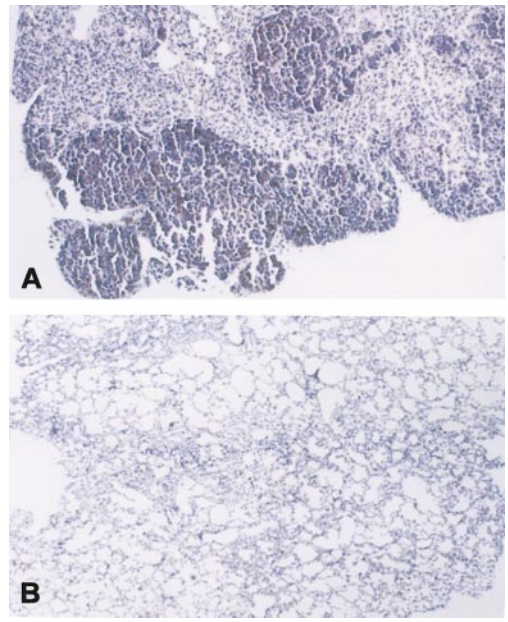

FIG. 5. Histochemical analysis of pulmonary metastatic B16 melanoma. Lung tissues isolated from the PBS- or salmosin-treated mice were fixed for 4 h in Bouin's solution before being embedded in paraffin. Sections were stained with hematoxylin and eosin. (A) PBS-treated control mouse. (B) Salmosin-treated mouse.

DISCUSSION

It is widely recognized that the two kinds of adhesion molecules, E-cadherin and integrins, play different but critical roles during tissue invasion and metastasis. Intercellular adhesion molecule called E-cadherin appears to help keep cells in place. Cell adhesion to ECM is mediated by cell-surface molecules known as integrins. Anchorage dependence allows cells to survive and proliferate.

Salmosin is a novel disintegrin containing RGD sequence that was discovered and characterized in our laboratory (17). It was demonstrated in the present work that recombinant salmosin expressed in *E. coli* strongly inhibits tumor cell adhesion, invasion through artificial ECM Matrigel, experimental tumor metasta-

sis, and tumor growth. In the present study, we have shown that salmosin strongly inhibits the metastasis of B16F10 melanoma cells by blocking the adhesion of the cells to ECM. And we observed that salmosin slightly inhibited the adhesion to laminin or type IV collagen. It is possible to postulate that the partial inhibitions of the adhesion to type IV collagen or laminin might be caused by structural differences between salmosin and RGD peptide that provided different affinities on various integrin receptors. In addition, other protein part of salmosin except RGD motif could be involved on the anti-adhesive activity of salmosin. The inhibition of the adhesion to collagen by salmosin may be due to blocking β1 integrin. Recent work in my laboratory showed that salmosin perturbed capillary endothelial cell adhesion to fibronectin, type I collagen by blocking β1 integrin (unpublished data). Therefore, we strongly suggested that salmosin is capable of acting as a potent antagonist of β1 integrin, as similar to other venom-derived disintegrins.

Furthermore, we observed that salmosin did not inhibit B16F10 melanoma cell proliferation onto type I collagen-coated plate at a concentration of approximately 1.0 μg/ml that showed a half-maximal inhibition of the cell adhesion. However, salmosin suppressed B16F10 melanoma cell proliferation at the concentration of 20 μg/ml that showed half-maximal inhibition of the cell proliferation. These results suggest that integrin αvβ3 or β1-mediated cell survival of B16F10 melanoma in collagen can be inhibited by salmosin, anti-αvβ3 monoclonal antibody or anti-β1 monoclonal antibody.

Several lines of experimental evidence clearly demonstrated that salmosin suppresses tumor metastasis. Therefore, based on the data presented here, it is possible to conclude that the suppression of the tumor metastasis led by salmosin is closely associated with the inhibited tumor cell adhesion to target organ and tumor cell proliferation via blocking integrin mainly for collagen.

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