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Cardiovascular Research Institute and BK21 Project for Medical Sciences, Yonsei University College of Medicine, Seoul 120-752, Korea

Kwang-Hoe Chung, Young-Doug Sohn, Yangsoo Jang

Department of Oncology, Graduate School of East-West Medical Science, Kyunghee University, Yongin 449-701, Korea

Sung-Hoon Kim, Kyu-yeon Han

Department of Biochemistry, College of Natural Science, Biotechnology Research Institute, Chungbuk National University, Chungjoo 361-763, Korea

Soo-Ik Chang

Cell and Gene Therapy Research Institute, College of Medicine, Pochon CHA University, CHA General Hospital, Seoul 135-081, Korea

Kwang-Hyun Baek

Department of Biochemistry, College of Science and Bioproducts Research Center, Yonsei University, Seoul 120-749, Korea

Doo-Sik Kim

Biotechnology Research Institute, Chungbuk National University, 48 San, Gashin-dong, Chungju 361-763, South Korea

In-Cheol Kang

Correspondence: I.-C. Kang, Biotechnology Research Institute, Chungbuk National University, 48 San, Gashin-dong, Chungju 361-763, South Korea. E-mail: ickang@khu.ac.kr

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Inhibitory effect of salmosin, a Korean snake venom-derived disintegrin, on the integrin α_v-mediated proliferation of SK-Mel-2 human melanoma cells

Kwang-Hoe Chung, Sung-Hoon Kim, Kyu-yeon Han, Young-Doug Sohn, Soo-Ik Chang, Kwang-Hyun Baek, Yangsoo Jang, Doo-Sik Kim and In-Cheol Kang

Abstract

We have investigated the inhibitory effect of salmosin on integrin-mediated human tumour cell proliferation. SK-Mel-2 human melanoma cell adhesion to denatured collagen or vitronectin was found to be significantly and statistically inhibited by salmosin in a dose-dependent manner ($P < 0.05$). Moreover, the binding of SK-Mel-2 cells to salmosin-coated plates was specifically disrupted by anti-integrin α_v monoclonal antibody at 8 μg mL^{-1}, but not by anti-integrin monoclonal antibody. These findings indicated that salmosin inhibited the adhesion of SK-Mel-2 cells to denatured collagen by specifically blocking integrin α_v. The proliferation of SK-Mel-2 cells on a denatured collagen-coated plate was statistically and significantly inhibited by salmosin induced apoptosis in a dose-dependent manner ($P < 0.05$). Anti-integrin α_v monoclonal antibody, anti-integrin $\alpha_v\beta_3$ monoclonal antibody, and synthetic RGD peptide also suppressed SK-Mel-2 cell proliferation. Several lines of experimental evidence strongly suggested that the inhibition of SK-Mel-2 cell proliferation by salmosin was due to the induction of apoptosis via the blocking of integrin α_v-mediated cell survival.

Introduction

Cell adhesion to extracellular matrix (ECM) components via integrins and syndecan molecules permits cell differentiation, proliferation, and survival by affecting cell cycle regulatory proteins. The ECM is a complex structure containing collagens, fibronectin, elastin, laminins, and glycoproteins (Gullberg et al 1992; Lukashev & Werb 1998; Aplin et al 1999; Giancotti & Ruoslahti 1999). The integrins act as transmembrane linkers between the ECM and the cytoskeleton by organizing structure and domain, which implies that the integrins play a critical role in outside–in signalling and inside–out signalling (Juliano & Haskill 1993). The interaction between integrins and cytoskeleton leads to the formation of focal adhesion complex that links species such as talin, α-actin, vinculin and tensin, and cellular molecules involved in signal transduction to the actin filament system (Ginsberg et al 1992; Burridge et al 1988). The clustering of integrins on the plasma membrane is initially triggered by the geometry of its binding sites in the ECM, and results in co-operative interactions between cytoskeleton elements connecting integrin cytoplasmic domains and actin filaments that are able to mediate stable attachment (Giancotti & Mainiero 1994).

Tumour cells can suppress apoptosis through a specific integrin–matrix interaction. Integrin $\alpha_v\beta_3$ expression has been closely related to tumorigenicity in malignant melanoma (Cheresh 1991; Felding-Habermann et al 1992). The integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ have been implicated in direct ligation to native type I collagen. Heat denatured type I collagen disrupts the binding to these integrins, and thus exposes cryptic RGD adhesive sites that can be ligated by integrin $\alpha v\beta_3$ (Davis 1992; Giancotti & Mainiero 1994; Petitclerc et al 1999). Proteolytic cleavage of type IV collagen also leads to the exposure of integrin $\alpha_v\beta_3$ cryptic binding sites, and thus contributes to angiogenesis and tumour growth (Xu et al 2001). The ligation of integrin $\alpha_v\beta_3$ within a three-dimensional

dermal collagen matrix was found to suppress apoptosis and to stimulate melanoma cell growth (Montgomery et al 1994). Recently, fibrillar collagen was reported to inhibit cell cycle progression, by adhesion through integrin $\alpha_2\beta_1$ and causing the upregulation of the cyclin inhibitor $p27^{KIP1}$ (Henriet et al 2000). These findings suggested that integrin-mediated adhesion to the ECM plays an important role in the survival of tumour cells.

Salmosin is a member of the disintegrin family, and inhibits platelet aggregation (Kang et al 1998), solid tumour growth by suppressing angiogenesis (Kang et al 1999) and tumour metastasis by disrupting integrin $\alpha_v\beta_3$-mediated adherence and proliferation (Kang et al 2000). These observations suggested that tumour growth inhibition by salmosin might be due to the suppression of integrin $\alpha_v\beta_3$-mediated tumour cell proliferation in addition to the inhibition of tumour-induced angiogenesis. In this paper, we have investigated whether the inhibition of human melanoma cell proliferation, on denatured collagen-coated plates, by salmosin, results in the induction of apoptosis by blocking integrin α_v.

Materials and Methods

Materials

Salmison was purified from recombinant *Escherichia coli* (Park et al 1998). SK-Mel-2 was obtained from the Korean Research Institute for Chemistry and Technology. B16F10 melanoma cells were obtained from the Mogam Biotechnology Research Institute, Korea. C57BL/6 mice were from Charles River, Japan. The following monoclonal antibodies (mAbs) against human integrin subunits and human vitronectin were purchased from Chemicon International (Temecula, CA): anti-integrin $\alpha 2$ (P1E6), $\alpha 3$ (P1B5), αv (AV1), $\beta 3$ (B3A), $\alpha_2\beta_1$ (BHA2.1), $\alpha_v\beta_3$ (LM609), and $\alpha_v\beta_5$ (P1F6). Two percent of gelatin solution was purchased from Sigma. The 2% denatured collagen solution, vitronectin, and annexin V were from Sigma, Chemicon International, and Pharmingen, respectively. The synthetic peptides, GRGDSP(Gly-Arg-Gly-Asp-Ser-Pro) and GRGESP (Gly-Arg-Gly-Glu-Ser-Pro) were from Peptron (Daeduk, Korea).

Cell adhesion assay

One hundred microlitres of solubilized denatured collagen (1.0 μg/well), vitronectin (1.0 μg/well) or salmosin (1.0 μg/well) in phosphate-buffered saline (PBS) was added to 96-well plates and incubated overnight at 4 °C. The plates were washed and incubated for 1 h with 0.5% bovine serum albumin to block unbound surface. The SK-Mel-2 human melanoma cells (5×10^4) were pre-incubated with salmosin, GRGDSP, GRGESP, or anti-integrin monoclonal antibodies for 20 min at 37 °C. The cells were then added to the wells and incubated for 1 h at 37 °C in 5% CO_2 and 95% air. Unattached cells were removed by washing with PBS and attached cells were fixed with methanol and stained with crystal violet. Absorbance of the individual well at 550 nm was measured by using an ELISA reader (Molecular Devices Corp., USA).

Cell proliferation assay

SK-Mel-2 human melanoma cells (8000 cells/well) were plated onto 24-well tissue culture plates, coated with denatured collagen (1.0 μg/well) and incubated in RPMI-1640 medium containing 5% foetal calf serum for 16 h. The integrin antagonists salmosin, anti-integrin mAbs, or synthetic RGD peptide were added to the cells. The experiment was performed in triplicate. After 72 h, adherent cells were dispersed in trypsin and counted.

Quantification of apoptotic cells

The cells plated on denatured collagen-coated wells were treated with salmosin or anti-integrin $\alpha_v\beta_3$ mAb for 48 h. After removing the culture media, attached cells were trypsinized, harvested, washed once in PBS at 4 °C and suspended in PBS. The cell suspension was incubated with annexin V-FITC and propidium iodide, according to the manufacturer's instructions (Vermes et al 1995), for 15 min in the dark. Analysis was by dual parameter flow cytometry.

Statistics

Numerical data are expressed as mean ± s.d. and statistical significance was calculated with data from at least three separate experiments. The mean inhibition of SK-Mel-2 cell proliferation and attachment were compared using the Kruskal–Wallis test. P values of less than 0.05 were considered to be statistically significant.

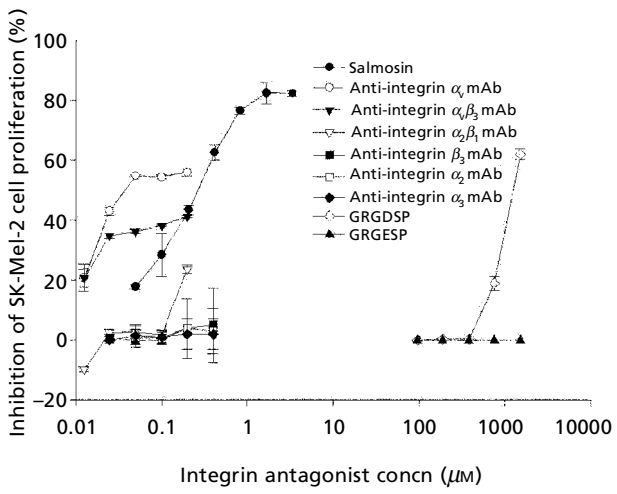

Figure 1 Inhibition of SK-MEL-2 melanoma cell proliferation on a denatured collagen-coated plate by salmosin and various integrin antagonists. SK-Mel-2 melanoma cells were incubated with salmosin and various monoclonal antibodies against integrin subunits in a 24-well plate coated with denatured collagen for 72 h. The wells were washed and attached cells were counted. Each point represents the mean of three determinations. Numerical data are mean ± s.d.

Results

Inhibition of SK-Mel-2 cell proliferation by salmosin

Our previous report suggested that salmosin inhibited tumour cell proliferation by disruption of integrin-mediated cell survival (Kang et al 1999). To assess the inhibitory effect of salmosin on tumour cell proliferation, we chose an in-vitro cell proliferation assay system and SK-Mel-2 human malignant melanoma cells. Salmosin was found to statistically and significantly inhibit SK-Mel-2 human melanoma proliferation on denatured collagen-coated plates in a dose-dependent manner ($P < 0.05$) (Figure 1). Half-maximal inhibition of cell proliferation by salmosin occurred at 2.0 $\mu g\,mL^{-1}$ (267 nM). Further investigation showed that anti-integrin α_v mAb (AV1), anti-integrin $\alpha_v\beta_3$ mAb (LM609), or synthetic RGD peptide (GRGDSP) also inhibited proliferation, but anti-integrin β_3 mAb (B3A), α_2 (P1E6), or α_3 (P1B5) had little or no effect. Salmosin appeared to most effectively inhibit SK-Mel-2 melanoma cell proliferation. These results suggested that an interaction between integrin α_v and salmosin resulted in the dose-dependent inhibition of SK-Mel-2 cell proliferation.

Inhibition of SK-Mel-2 cell adhesion to denatured collagen by salmosin

Cell adhesion to denatured collagen is mediated by the $\alpha_v\beta_3$, $\alpha_2\beta_1$ or $\alpha_3\beta_1$ integrins. To determine whether the inhibition of SK-Mel-2 cell proliferation by salmosin was due to the blocking of integrin-mediated cell adhesion to denatured collagen, we identified the integrin subunits involved in this cell adhesion. By in-vitro cell adhesion assay, salmosin was found to statistically and significantly inhibit SK-Mel-2 cell adhesion to denatured collagen or vitronectin in a dose-dependent manner ($P < 0.05$) (Figure 2). The half-maximal cell adhesion inhibition was observed at salmosin concentrations of approximately 80 and 133 nM on denatured collagen and vitronectin, respectively (Figure 2). To determine which integrin subunits were specifically blocked by salmosin, we performed an in-vitro cell adhesion assay. Premixed SK-Mel-2 human melanoma cells and anti-integrins α_v, α_2, $\alpha_v\beta_3$, $\alpha_2\beta_1$ or β_3 mAb were applied to 96-well plates coated with salmosin. Interestingly, anti-integrin α_v mAb markedly suppressed cell adhesion to salmosin, but anti-integrin $\alpha_2\beta_1$ and β_3 mAbs had no effect (Figure 3). Taken together, these results demonstrated that salmosin inhibited SK-Mel-2 cell adhesion to denatured collagen by disrupting the action of integrin α_v.

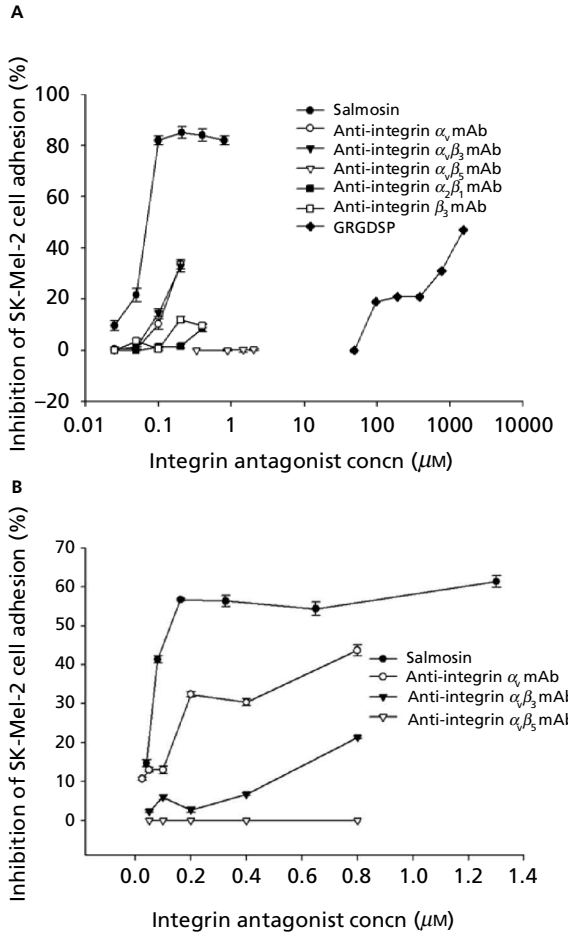

Figure 2 Inhibition of SK-Mel-2 cell attachment to denatured collagen (A) and vitronectin (B) by salmosin and various integrin antagonists. SK-Mel-2 cells were pre-incubated with salmosin or various integrin antagonists for 30 min before being added to denatured collagen- and vitronectin-coated wells. The wells were washed after 1-h incubation and attachment was determined colorimetrically. Each point represents the mean of three determinations. Numerical data are mean ± s.d.

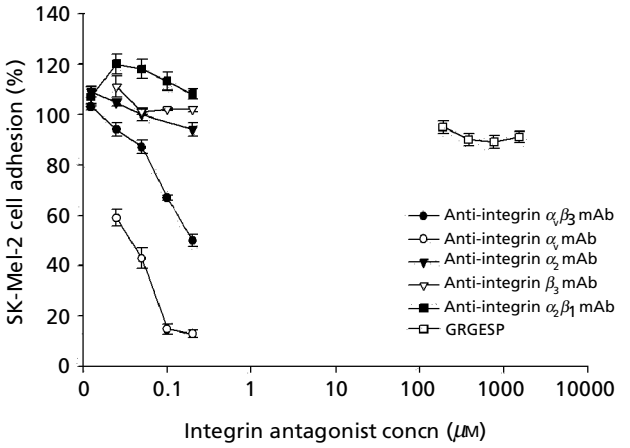

Figure 3 Effects of various integin antagonists on SK-Mel-2 cell attachment to salmosin. SK-Mel-2 cells were pre-incubated with integrin antagonists for 30 min and then plated in salmosin-coated wells. After incubating for 1 h, cells were fixed and adhesion was measured as described in Figure 2. Each point represents the mean of three determinations. Numerical data are mean ± s.d.

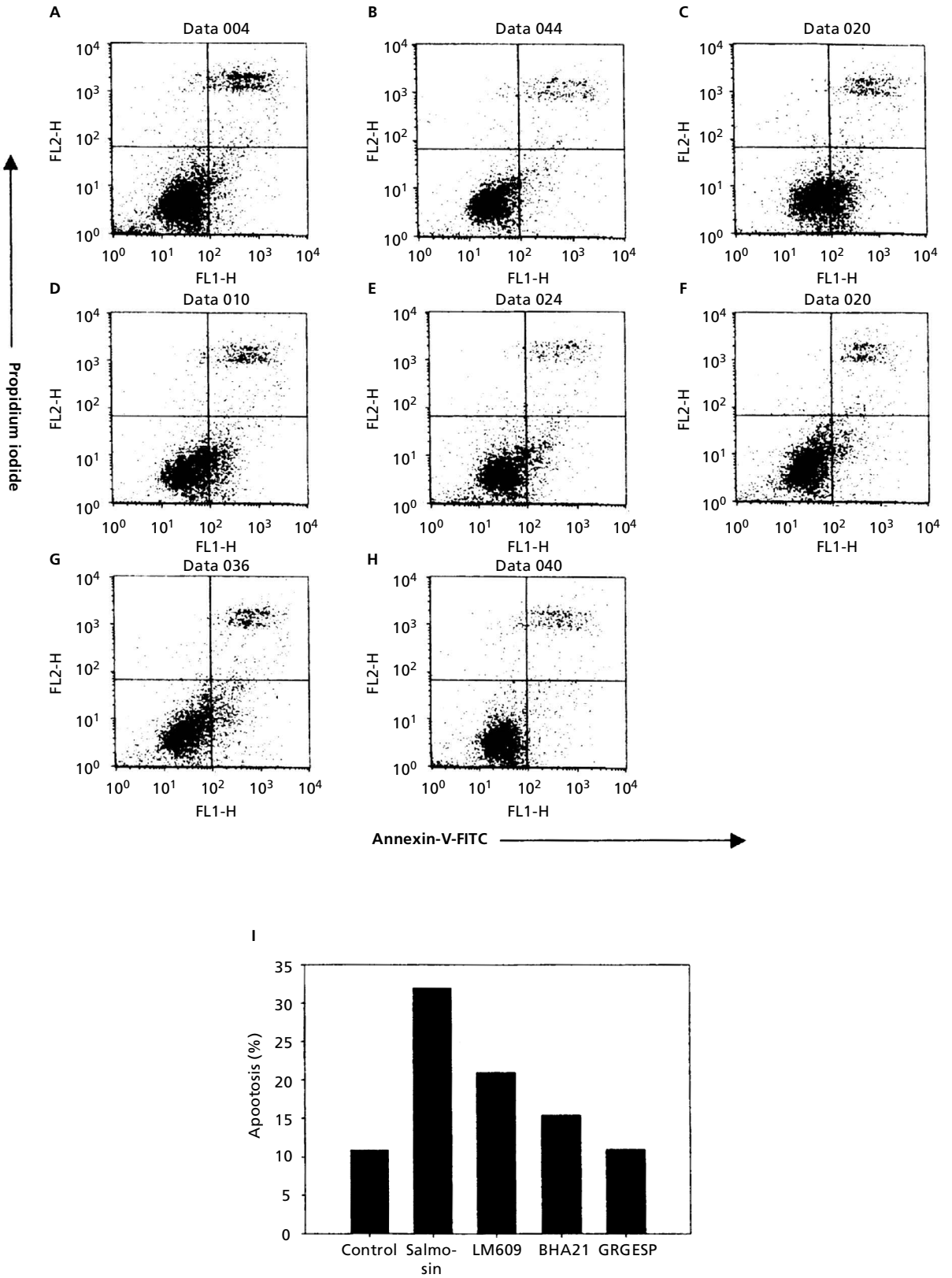

Figure 4 Effect of integrin antagonists on the induction of apoptosis in SK-Mel-2 melanoma cells. A, A sample-free culture medium blank control. B, GRGESP 1.0 mg mL^{-1}; C, salmosin 12.5 μg mL^{-1}; D, salmosin 6.25 μg mL^{-1}; E, anti-$\alpha_2\beta_1$ mAb (BHA2.1) 25 μg mL^{-1}; F, anti-$\alpha_2\beta_1$ mAb (BHA2.1) 12.5 μg mL^{-1}; G, anti-$\alpha_v\beta_3$ mAb (LM609) 25 μg mL^{-1}; or H, anti-$\alpha_v\beta_3$ mAb (LM609) 12.5 μg mL^{-1} were added to cells and incubated for 48 h. After the incubation, cells attached to the plates were harvested and stained with annexin-V and propidium iodide. I represents fluorescence intensities corresponding to annexin-V-FITC in salmosin (12.5 μg mL^{-1}), LM609 (25 μg mL^{-1}), BHA2.1 (25 μg mL^{-1}), and GRGESP (1.0 mg mL^{-1})-treated cells.

Salmosin-induced apoptosis in SK-Mel-2 melanoma cells

Integrin $\alpha_v\beta_3$ plays an important role in cell proliferation and survival. To investigate whether the inhibitory effect of salmosin on cell proliferation may be due to apoptosis, we performed annexin-V staining in-vitro. Cells treated with salmosin, anti-integrin α_v mAb, or anti-integrin $\alpha_2\beta_1$ mAb were incubated with FITC-labelled-annexin V and apoptosis levels were determined by measuring fluorescence. Salmosin enhanced the percentage of apoptotic cells on denatured collagen in a dose-dependent manner. Thirty-two percent of salmosin-treated cells showed apoptosis at 0.84 μM whereas only 10% salmosin untreated cells were apoptotic. In addition, 21% of cells treated with anti-integrin $\alpha_v\beta_3$ (LM609) were apoptotic (Figure 4). These results strongly suggested that apoptotic induction in SK-Mel-2 cells on denatured collagen by salmosin was probably due to integrin α_v suppression, which agreed with the cell adhesion assay finding described above, and suggested that salmosin specifically blocked integrin α_v.

Discussion

We demonstrated previously that salmosin inhibited tumour progression by suppressing angiogenesis by blocking integrin $\alpha v\beta_3$ (Kang et al 1999). Salmosin was reported to have anti-metastatic functionality in B16 melanoma cells (a metastasis model) by disrupting integrin-mediated cell adhesion and invasion (Kang et al 2000). The α_v integrin subunit is a viable cancer therapy target, because vitronectin receptors, such as integrin $\alpha_v\beta_3$, play a pivotal role in melanoma cell growth and invasion (Marshall et al 1991; Gehlsen et al 1992). In this study, we found that salmosin suppressed B16 melanoma cell proliferation on denatured collagen or type I collagen, though salmosin inhibited their proliferation only marginally on polylysine (unpublished data). Moreover, Kang et al (1999) found that the anti-angiogenic function of salmosin may be due to the prevention of integrin $\alpha_v\beta_3$-mediated endothelial cell proliferation and adhesion. A partially phosphorothioated antisense oligonucleotide targeting the integrin α_v gene was found to induce apoptosis in human breast carcinoma cells (Townsend et al 2000). Two antagonists of integrin $\alpha_v\beta_3$, a human specific monoclonal antibody (17E6), and a cyclic RGD peptide were found to inhibit human melanoma growth in-vivo (Mitjans et al 2000). In this study, we also found that salmosin was able to inhibit the proliferation of SK-Mel-2 human melanoma cells by inducing apoptosis (Figures 1 and 4). Interestingly, cells attached to denatured collagen were induced to apoptosis by salmosin or anti-α_v mAb treatment. These results showed that salmosin or anti-α_v mAb induced apoptosis in human melanoma cells by inhibiting integrin α_v. Anti-$\alpha_v\beta_3$, -$\alpha_2\beta_1$, -α_v mAb, and GRGDSP suppressed SK-Mel-2 cell proliferation, but anti-α_2 and -β_3 mAb were ineffective in this context. Notably, SK-Mel-2 cell adhesion to salmosin was found to be specifically interrupted by anti-α_v mAb, indicating that salmosin may selectively target integrin α_v, rather than the other integrin subunits expressed on the cellular surface (Figure 3). Thus, we believe it likely that salmosin may block the α_v integrin series, which play an important role in tumour invasion and progression, via the RGD motif, a well-known binding site for integrin β subunit and other subunits. Therefore, we suggest that the inhibition of tumour growth by salmosin may be a consequence of the prevention of tumour-induced angiogenesis by integrin $\alpha_v\beta_3$-blockage, and by the suppression of tumour cell growth by specifically inhibiting integrin α_v-mediated proliferation.

Conclusion

The anti-angiogenic and anti-metastatic properties of the disintegrin, salmosin, derived from the venom of the Korean snake, *Agkistrodon halys brevicaudus*, were found to be due to its blocking of integrin receptors, including integrin $\alpha_v\beta_3$. In this study, we found that the anti-proliferative effect of salmosin on SK-Mel-2 human melanoma cells occurred because it specifically disrupted the functionality of integrin α_v on the cellular surface. Moreover, tumour cell apoptotic induction by salmosin was identified as a possible mechanism of tumour growth inhibition. Our results showed that salmosin may represent a developmental base for future anti-tumour agents.

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