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Inhibition of integrins αv/α5-dependent functions in melanoma cells by an ECD-disintegrin acurhagin-C

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ABSTRACT

Acurhagin-C, a Glu–Cys–Asp (ECD)-disintegrin from *Agkistrodon acutus* venom, has been reported as an endothelial apoptosis inducer, previously. Here we further evaluate its potential applications in cancer therapy. In vitro assays indicated that acurhagin-C not only may influence the cell viability at higher concentration, but also can potently and dose-dependently decrease cell proliferation in murine B16-F10 melanoma. Otherwise, it also had a dose-dependent inhibition on B16-F10 cell adhesion to extracellular matrices, collagen VI, gelatin B and fibronectin, as well as disturbed transendothelial migration of B16-F10 cell. Morphological study found that acurhagin-C dramatically affected B16-F10 cell adhesion to immobilized fibronectin, leading to the formation of multicellular aggregates with rounded shape. Detected by flow cytometry, acurhagin-C was able to induce B16-F10 cell apoptosis and alter cell cycle distribution through its interactions with integrins αv/α5, and thereafter initiation the apoptotic pathways of caspase-8/-9. Furthermore, acurhagin-C could synergistically enhance the anti-proliferative activity of methotrexate in B16-F10 cells and human melanoma SK-MEL-1 cells, without diminishing the growth of human epidermal melanocytes. Taken together, acurhagin-C proved to be a potent inhibitor of integrin-based functions in melanoma cells by activating the complex apoptotic pathways.

1. Introduction

The interactions of cells with neighboring cells and the surrounding extracellular matrices (ECMs) are mediated by different classes of cell adhesion receptors. Integrins, a family of heterodimeric transmembrane adhesion receptors linked by the non-covalent association of α- and β-subunits, primarily mediate the interactions of cells with ECMs and play critical roles in promoting cell survival, proliferation, and migration in vitro and in vivo (Hynes, 1999). Interference of integrin-mediated functions may suppress tumor metastasis and induce tumor apoptosis. Therefore, integrin antagonists have therapeutic applications in the treatment of cancer.

Integrins not only can mediate cell adhesion to ECMs but also transduce intracellular signals that promote cell survival (Meredith et al., 1993) and migration (Schwartz and Shattil, 2000). The association of integrins with crosslinked ECMs cluster integrins and their associated cofactors may lead to the initiation of integrin-modulated signaling pathways. For example, integrin ligation prevents cell undergoing apoptosis by activating suppressors of apoptosis (Pankov et al., 2003) and by inhibiting caspase activation (Stupack et al., 2001; Kim et al., 2002). Furthermore, these adhesion molecules also promote cell cycle entry by stimulating expression of cyclins (Assoian and Schwartz, 2001). Although integrin ligation can initiate signal transduction cascades, inhibiting integrin-ligand interactions may affect cell proliferation and induce apoptosis (Bakre et al., 2002; Jin and Varner, 2004).

Disintegrins are a family of low molecular mass, cysteine-rich, naturally occurring polypeptides from a variety of *Viperidae* and *Crotalidae* snake venoms. They mostly contain an Arg–Gly–Asp (RGD) sequence that bind to integrins on the surface of normal and malignant cells (Dennis et al., 1990; Scarborough et al., 1993). Numerous disintegrins exert RGD-tripeptide acting as a critical recognition sequence, whereas some heterodimeric disintegrins express integrin-binding motifs other than the RGD-tripeptide, such as lebestatin and jerdostatin (Walsh and Marcinkiewicz, 2011). RGD-disintegrins have been characterized from various snake venoms and were originally characterized as platelet aggregation inhibitors (Eble, 2010). Interestingly, disintegrins have been proven to bind to various integrins with high affinity and always inhibit the binding of the natural ligand. Disintegrins are highly homologous, while differences also exist in their affinity and selectivity for integrins (van der Flier and Sonnenberg, 2001).

According to the length of polypeptide chain and number of disulfide bonds, disintegrins have been classified into five groups including short-disintegrin (e.g., echistatin), medium-disintegrins (e.g., kistrin),

Abbreviations: ECD, Glu–Cys–Asp; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; HUVECs, human umbilical vein endothelial cells; MFI, mean fluorescence intensity; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide; MTX, methotrexate; PE, phycoerythrin; RGD, Arg–Gly–Asp.

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long-disintegrins (e.g., arietin), dimeric disintegrins (e.g., lebestatin), and disintegrins (e.g., catrocollastatin-C) degraded from the C-termini of protein class III snake venom metalloproteinases (SVMPs) (Calvete et al., 2005; Swenson et al., 2007; Calvete et al., 2009). Most reported monomeric disintegrins have an RGD- or RGD-like tripeptide located at the tip of a loop formed by disulfide bond that is responsible for their interactions with various integrins (Calvete et al., 2005). Previously, a Glu-Cys-Asp (ECD)-containing disintegrin acurhagin-C, purified from Formosan *Agkistrodon acutus* venom, has been identified as an endothelial apoptosis inducer (Selistre-de-Araujo et al., 2010; Wang, 2010).

Integrins have been demonstrated to play a critical role for the proliferative, adhesive and migratory properties of tumor cells. Thus, any disintegrin that can interfere with these processes may possibly be used in the treatment of tumor growth and metastasis. In this study, we further examined the biological activities and molecular mechanism of acurhagin-C in murine B16-F10 melanoma cells, a highly metastatic carcinoma cell line (Albini et al., 1987). In addition, the efficacy and selectivity of acurhagin-C on integrins-mediated proliferation in human melanoma and human melanocytes were also investigated.

2. Results

2.1. Effects of acurhagin-C on the viability and proliferation of cultured B16-F10 cell

Acurhagin-C, derived from the C-terminal 23.5-kDa fragment of a metalloproteinase acurhagin, is composed of an ECD-containing disintegrin-like domain and a cysteine-rich domain (Wang, 2010). Our preliminary experiments found that acurhagin-C is not only able to affect the attachment of suspended B16-F10 cells but also induce the detachment of adhered B16-F10 cells. Therefore, in vitro assay of acurhagin-C on the viability of B16-F10 cell was carried out. After serum deprivation, the growth of cultured B16-F10 cells is clearly reduced as comparison with the case of 10% FBS-treated cells (Fig. 1A). However, the RGD-disintegrin kistrin (0.2 μM), cyclic RGD-peptide cRGDfV (30 μM) and acurhagin-C (0.04 and 0.2 μM) all exhibited no effect on the viability of B16-F10 cells in serum-free medium for 44 h-incubation. These results indicated that acurhagin-C, similar to kistrin and cRGDfV, had no effect on melanoma cell viability. In contrast, acurhagin-C at 0.8 μM significantly decreased the viability of B16-F10 cells by 20%.

It has been reported that a variety of RGD-disintegrins, such as kistrin and triflavin, are effective anti-tumor agents via their specific blockade of integrins (McLane et al., 2004). To evaluate the therapeutic potential of acurhagin-C in highly metastatic cell, the proliferation assay of B16-F10 cells was then performed. As shown in Fig. 1B, the RGD-peptides cRGDfV showed a little effect on B16-F10 cells proliferation, even at the higher concentration of 30 μM. However, both integrins, acurhagin-C (0.04–0.8 μM) and kistrin (0.2 μM), had a significant suppression on the growth of B16-F10 cells. Based on this evidence, acurhagin-C seems to be a potent inhibitor of melanoma cell proliferation.

2.2. Effects of acurhagin-C on B16-F10 cell adhesion to ECM and transmigration into fibronectin-coated membrane

Integrins are the major mediators for cell adhesion to ECMs, such as collagens and fibronectin. In fact, adhesion affects many aspects of cell behavior. Particularly important is the influence of adhesion on cell survival (Burridge and Tsukita, 2001). To investigate the effect of acurhagin-C on B16-F10 cells adhesion to ECMs, ninety-six-well microplates were pre-coated with various matrices for the used in adhesion assay. As shown in Fig. 2A, acurhagin-C inhibited B16-F10 cells attachment to collagen VI, gelatin B, and fibronectin, in a

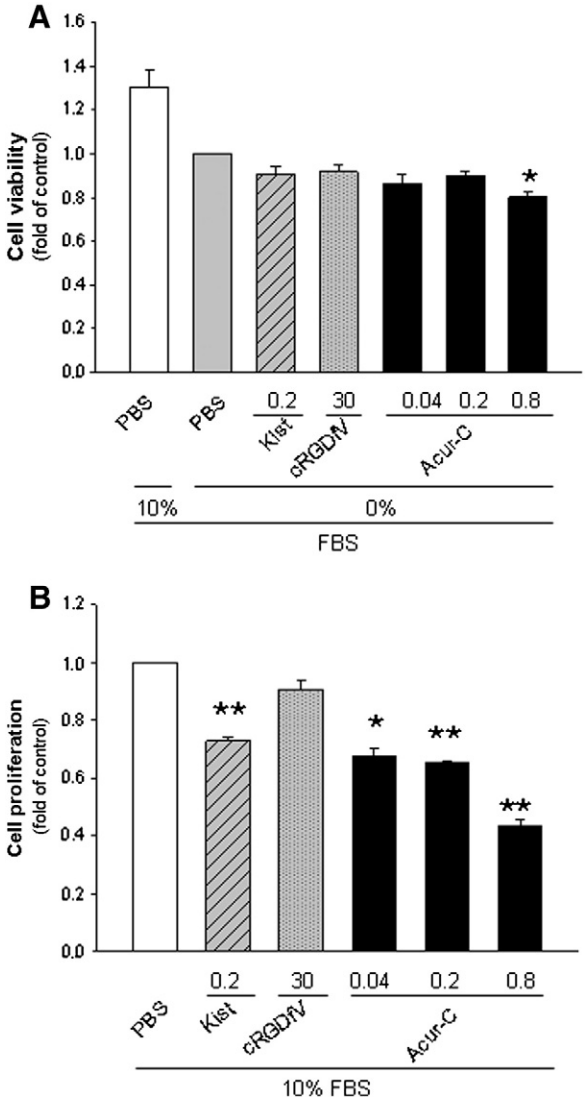

Fig. 1. Effects of acurhagin-C on B16-F10 cells viability and proliferation. After pretreatment with PBS, an RGD-disintegrin (kistrin, Kist; 0.2 μM), a synthetic cyclic RGD-peptide (cRGDfV; 30 μM), and acurhagin-C (Acur-C; 0.04, 0.2 and 0.8 μM) for 15 min at RT, B16-F10 cells (1×10^4 cells per well) in 10 % FBS or serum-free/DMEM (A) and 10 % FBS/DMEM (B) were seeded onto 96-well plates at 37 °C/5% CO_2 for 44 h and 24 h, respectively, and followed by MTT assay. All experiments were performed in quadruplicate and similar results were repeated at least three times. Results are expressed as relative activity of acurhagin-C on the inhibition of viability and proliferation of B16-F10 cells (560 nm), respectively, and presented as mean ± SEM ($n=3$). $^*P<0.01$, $^{**}P<0.001$ as compared with control.

concentration-dependent manner with IC_{50} values of approximately 0.15, 0.34 and 0.65 μM, respectively. Acurhagin-C, therefore, also has an inhibitory effect on the adhesion of melanoma cells to ECMs.

Fibronectin, a major extracellular ligand, is capable of recognizing nine RGD-dependent integrins and three non-RGD-dependent integrins (Plow et al., 2000). For evaluating the anti-metastatic potential of acurhagin-C, fibronectin was pre-coated on the filter membrane in Boyden chamber. Transendothelial migration assay was subsequently performed. Migratory B16-F10 cells were quantified by a colorimetric method. By contrast to the chambers without B16-F10 cells added, B16-F10 cells markedly migrated to the underside of filter membrane (Fig. 2B). However, acurhagin-C (0.8 μM), but not BSA, significantly interfered with the transendothelial migration of B16-F10 cells.