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Alternagin-C, a disintegrin-like protein from the venom of *Bothrops alternatus*, modulates $\alpha_2ß_1$ integrin-mediated cell adhesion, migration and proliferation

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Abstract

The $\alpha_2ß_1$ integrin is a major collagen receptor that plays an essential role in the adhesion of normal and tumor cells to the extracellular matrix. Alternagin-C (ALT-C), a disintegrin-like protein purified from the venom of the Brazilian snake *Bothrops alternatus*, competitively interacts with the $\alpha_2ß_1$ integrin, thereby inhibiting collagen binding. When immobilized in plate wells, ALT-C supports the adhesion of fibroblasts as well as of human vein endothelial cells (HUVEC) and does not detach cells previously bound to collagen I. ALT-C is a strong inducer of HUVEC proliferation *in vitro*. Gene expression analysis was done using an Affimetrix HU-95A probe array with probe sets of ~10,000 human genes. In human fibroblasts growing on collagen-coated plates, ALT-C up-regulates the expression of several growth factors including vascular endothelial growth factor, as well as some cell cycle control genes. Up-regulation of the vascular endothelial growth factor gene and other growth factors could explain the positive effect on HUVEC proliferation. ALT-C also strongly activates protein kinase B phosphorylation, a signaling event involved in endothelial cell survival and angiogenesis. In human neutrophils, ALT-C has a potent chemotactic effect modulated by the intracellular signaling cascade characteristic of integrin-activated pathways. Thus, ALT-C acts as a survival factor, promoting adhesion, migration and endothelial cell proliferation after binding to $\alpha_2ß_1$ integrin on the cell surface. The biological activities of ALT-C may be helpful as a therapeutic strategy in tissue regeneration as well as in the design of new therapeutic agents targeting $\alpha_2ß_1$ integrin.

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Introduction

Cell attachment to the extracellular matrix (ECM) is mediated primarily by the integrins, a large family of glycoproteins expressed on the cell surface (1). Integrins are heterodimers formed of non-covalently linked α- and ß-subunits (2). Usually, integrin-mediated cell adhesion results in the activation of intracellular signaling pathways due to the association of many different signaling proteins at the focal contact sites (3). Aggregation of integrin receptors, association of cytoskeleton proteins, and tyrosine kinase-mediated phosphorylation are key events responsible for diverse cell responses such as cell migration and differentiation, tissue remodeling, cell proliferation, angiogenesis, and tumor cell invasion and metastasis (1,4).

Cell adhesion to the ECM is partially mediated by the binding of integrin to an integrin-recognition RGD motif found in some ECM components such as fibronectin, vitronectin and fibrinogen (5). This motif is also found in a group of small cysteine-rich proteins found in some snake venoms named disintegrins (6). Disintegrins inhibit cell-matrix and cell-cell interactions mediated by integrins (7,8). Most disintegrins are very potent inhibitors of platelet aggregation by acting as antagonists of the fibrinogen receptor, $\alpha_{IIb}ß_3$ integrin. This activity is due to the presence of the adhesive RGD motif within an amino acid hairpin loop maintained by disulfide bridges (9). In addition to inhibiting platelet aggregation, some disintegrins have also been shown to inhibit experimental metastasis as an integrin-dependent process, and therefore, over the last few years, many studies have focused on these proteins (10,11).

A different class of disintegrins is also found in snake venom that does not contain the RGD motif. These proteins are larger than the RGD disintegrins (about 30 kDa) and they have an extra C-terminal, cysteine-rich domain (12-15). These disintegrins do not bind to $\alpha_{IIb}ß_3$, $\alpha_5ß_1$ or $\alpha_vß_3$ integrins, but interact with the collagen receptor, $\alpha_2ß_1$ integrin, therefore inhibiting cell adhesion to collagen I. The D/ECD sequence replaces the RGD motif, and it has been suggested that this sequence is involved in integrin binding.

Most of the RGD and non-RGD disintegrins are synthesized in the venom gland as precursor forms having pro- and metalloproteinase domains, and proteolytic processing of these proteins releases the disintegrin-like/cysteine-rich domain (6,12,13,16). Homologous proteins (the ADAMs, for a disintegrin and metalloproteinase) are found in mammals as well as in several other organisms, in which they are involved in several physiological processes such as fertilization, cell differentiation, and shedding of receptors (17). The ADAMs have a similar domain organization with extra-domains including transmembrane and intracellular domains (18). Both ADAMs and snake venom metalloproteinase belong to the reprolysin protein family of metalloproteinases (19).

We have described the isolation and characterization of alternagin-C (ALT-C), a disintegrin-like protein from *Bothrops alternatus* snake venom (14). ALT-C is synthesized as a precursor form with a metalloproteinase domain from which it is released after proteolytic processing, yielding a form with disintegrin- and cysteine-rich domains (14). Here we will review the major findings that have been reported for this disintegrin-like protein, focusing on its effects on cell adhesion, migration and proliferation.

Isolation and characterization of alternagin-C

ALT-C was purified from *Bothrops alternatus* venom by two steps of gel filtration followed by anion exchange chromatography (14). The molecular mass of the purified protein was estimated at 29 kDa by SDS-PAGE and the protein had no demonstrable

enzymatic activity. Its partial amino acid sequence was determined by Edman degradation on an automated protein sequencer and the protein was shown to have the ECD motif (14). The partial amino acid sequence of ALT-C confirmed its homology with the disintegrin-like proteins.

Effects of ALT-C on cell adhesion

Inhibition of cell adhesion

Adhesion studies are usually carried out in 96-well plates previously coated with adhesion molecules such as fibronectin, collagen or vitronectin. Cells are incubated on these plates in the presence or absence of the disintegrin. We demonstrated that ALT-C is a potent inhibitor of collagen binding to this integrin using an erythroleukemia cell line (K562) transfected with the $\alpha_2\beta_1$ integrin. Its ability to inhibit collagen-induced adhesion was dose-dependent and specific for cells expressing $\alpha_2\beta_1$ integrin. ALT-C did not interfere with the adhesion of cells expressing $\alpha_{IIb}\beta_3$, $\alpha_1\beta_1$, $\alpha_5\beta_1$, $\alpha_4\beta_1$, $\alpha_V\beta_3$, and $\alpha_9\beta_1$ integrins to other ligands such as fibrinogen, fibronectin, collagen IV, and vascular cell adhesion molecule 1 (14). Even the main collagen type IV receptor, integrin $\alpha_1\beta_1$, was not affected by ALT-C.

ALT-C also inhibited the adhesion of mouse fibroblasts to collagen I (20) and several tumor cell lines such as human cervix epithelioid carcinoma (HeLa), human bladder epithelioid carcinoma (ECV-304/T24), and the estrogen-independent breast human carcinoma (MDA-MB-231) (Terruggi CHB, Bérard M, Crépin M and Selistre-de-Araujo HS, unpublished data). These results suggest that the $\alpha_2\beta_1$ integrin is one of the major collagen receptors in these cells.

Support of cell adhesion

ALT-C is also considered to be an adhesion molecule itself. In 96-well plates coated with ALT-C, this disintegrin significantly supported the adhesion of mouse fibroblasts, human vein endothelial cells (HUVEC) (20), and $\alpha_2\beta_1$-transfected K562 cells (14).

Some RGD disintegrins can detach cells bound to adhesion molecules (21). Cell detachment usually results in "anoikis", a form of apoptotic cell death that occurs upon loss of matrix attachment (22). However, ALT-C does not detach cells bound to collagen I, gelatin or fibronectin-coated surfaces (20). Thus, ALT-C strongly favors cell adhesion, acting as a survival factor.

Effects of ALT-C on cell proliferation

HUVECs were incubated for 72 h at 37ºC in 199 medium plus 5% FBS in the presence of ALT-C. Cell concentration was measured by the MTT method (20). ALT-C both immobilized in plastic wells and soluble induced endothelial cell proliferation *in vitro* in a dose-dependent manner (20). The dose-response curve was bell-shaped, with concentrations ranging from 1 to 40 nM inducing proliferation after 72-h incubation, whereas at higher concentrations such as 100 nM, ALT-C had the opposite effect, inhibiting HUVEC proliferation. It has been reported that $\alpha_2\beta_1$ integrin plays a major role in endothelial cell proliferation (23). Therefore, ALT-C can bind to this integrin, which triggers the activation of intracellular signaling pathways leading to cell proliferation. ALT-C strongly activates Akt/PKB phosphorylation in HUVECs, an essential signaling pathway for endothelial cell proliferation which is activated by many angiogenic factors (20,24).

This effect seems to be specific for cells expressing significant levels of $\alpha_2\beta_1$ integrin. ALT-C significantly increased the proliferation in MDA-MB-231 cells which have been described as a highly migratory and invasive cell line that produces significant amounts of $\alpha_2\beta_1$ integrin (Terruggi CHB, Bérard M, Crépin M and Selistre-de-Araujo

HS, unpublished data). However, human and mouse fibroblasts were not sensitive to ALT-C in terms of proliferation, and the same was observed for some tumor cell lines such as HeLa and ECV-304. Maybe these cell lines express lower levels of $\alpha_2\beta_1$ integrin but further comparative studies are needed in order to quantify the expression levels of this integrin in these cell lines to obtain a better understanding of the proliferative effect ALT-C.

Effects of ALT-C on gene expression

For a better understanding of the mechanism of action of ALT-C, we studied the pattern of gene expression in fibroblasts treated with this disintegrin. For this purpose, plate wells were coated with ALT-C and human fibroblasts were incubated for 2 h at 37ºC. After this time, total RNA was extracted and used in a GeneChip hybridization experiment carried out with an Affimetrix HU-95A probe array containing probe sets representing ~10,000 human genes (20).

Under these conditions, ALT-C induced a significant increase in several genes related to cell cycle control, including vascular endothelial growth factor (VEGF) and other growth factors such as inducible early growth response, interleukin 11, early growth response 2 and 3, and insulin-induced gene. The expression of VEGF may explain the positive effect of ALT-C on HUVEC proliferation.

VEGF is a cytokine essential for the vasculogenesis associated with normal embryonic development and for the angiogenesis associated with wound healing, cancers, and a variety of other important pathologies. VEGF exerts multiple effects on the vascular endothelium including the stimulation of endothelial cell proliferation, rapid induction of microvascular permeability, promotion of endothelial cell survival, stimulation of endothelial cell adhesion and migration, and induction of endothelial cell gene expression (25). The proliferative effect of ALT-C alone on endothelial cells was similar to that exerted by VEGF and fibroblast growth factor 2, and the presence of ALT-C partially inhibited the endothelial cell proliferation induced by VEGF and fibroblast growth factor 2 (20).

VEGF expression induced by ALT-C was confirmed by ELISA in a different experiment in which fibroblasts growing on collagen I were treated with soluble ALT-C. After 24 and 48 h of incubation, VEGF levels were strongly increased in the culture supernatants compared to untreated cells (20).

As far as we know, ALT-C is the only disintegrin reported to induce VEGF expression and promote HUVEC proliferation. Most studies have been done on RGD disintegrins which induce endothelial cell apoptosis and inhibit angiogenesis (26-28). Aggretin, a potent platelet-aggregating protein purified from *Calloselasma rhodostoma* venom, which consists of α and ß subunits sharing homologous sequences to those of C-type lectins, is one of the rare examples of a venom protein that elicits VEGF expression resulting in HUVEC proliferation and migration and promoting angiogenesis *in vivo* and *in vitro* (29). Some VEGF-like proteins have also been found in Viperidae snake venoms, whose role in envenomation was thought to be due to the increase in vascular permeability and subsequent shock (30,31).

Effects of ALT-C on neutrophil signaling

Chemotaxis is determined in a 48-well Boyden chamber with a 5-µm pore filter and using human neutrophils. ALT-C has a potent chemotactic effect on human neutrophils, comparable to that of *N*-formyl-methionyl-leucyl-phenylalanine peptide, a classic chemotactic agent (32). A significant increase in F-actin content is observed in cells treated with ALT-C, showing that the chemotactic activity of

ALT-C is driven by dynamic changes in the actin cytoskeleton. Furthermore, ALT-C induces an increase in phosphotyrosine content by triggering focal adhesion kinase activation and its association with phosphatidylinositol 3-kinase. ALT-C also induces a significant increase in extracellular signal-regulated kinase 2 nuclear translocation (32). These findings suggest that this disintegrin can induce neutrophil migration modulated by intracellular signals characteristic of integrin-activated pathways. Again, $\alpha_2\beta_1$ integrin may be directly involved in this effect of ALT-C since this receptor has been described as one of the major receptors for neutrophil chemotaxis (33).

Effects of ALT-C on tumor cell migration

Cell migration experiments were performed using Transwell® plates containing an 8-µm pore filter coated or not with collagen I (10 µg/mL). In the presence of collagen I, ALT-C (0.1-10 nM) stimulates the transmigration of MDA-MB-231 cells and at higher concentrations (10-1000 nM) it has the opposite effect, inhibiting the cell movement toward collagen. However, in the absence of collagen I, there is no stimulation. For the MCF-7 cell line, there is no significant effect with or without the presence of collagen I (Terruggi CHB, Bérard M, Crépin M and Selistre-de-Araujo HS, unpublished data). The presence of collagen I in the assay was essential for the effect of disintegrin, probably due to the activation of integrin, which is needed before ligand recognition.

Lundström et al. (34) have demonstrated an important role of the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ collagen receptors during the initial attachment of MDA-MB-231 human breast cancer cells to extracellular bone and lung matrices. They correlated the expression of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins with the ability of different cancer cell types to bind to cortical bone. Since type I collagen is the major protein present in cortical bone, a potential effect of ALT-C in preventing bone metastasis is suggested for treatment of tumor cells expressing $\alpha_2\beta_1$ integrin.

Concluding remarks

ALT-C can competitively bind and activate $\alpha_2\beta_1$ integrin, with the activation of a signaling pathway that leads to cell migration and/or proliferation in cells expressing large amounts of $\alpha_2\beta_1$ integrin such as neutrophils and HUVEC. Thus, ALT-C may be considered an interesting tool for cell biology studies as well as for future therapeutic applications targeting $\alpha_2\beta_1$ integrin. The interaction of ALT-C with a signaling pathway for integrin activation in some cancer cell types interferes with its migration and adhesion mechanisms, and these effects can be modulated depending on the disintegrin concentration. It may be interesting to stimulate HUVEC proliferation during wound healing and tissue regeneration.

Members of the ADAM protein family in mammals and other species bind to several integrins and also support cell adhesion through the disintegrin/cysteine-rich domains (35,36). However, very little is known about intracellular signaling or gene expression mediated by the interaction of ADAMs and integrins. Given the homology of snake venom disintegrin-like proteins and the disintegrin domains of the members of the ADAM protein family, the results obtained for ALT-C suggest that similar functions could be assigned to the latter, such as signaling via integrins leading to key events like gene expression and affecting cell proliferation.

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