New Insights on Disintegrin-Receptor Interactions: Eristostatin and Melanoma Cells

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Key Words

Integrin · Disintegrin · Eristostatin ·
Melanoma · Metastasis · NK cells

Abstract

Viper venom disintegrins have been used frequently to study the cellular receptors which characterize various types of cells, including platelets, endothelial cells and cancer cells. While the majority of such analyses have pointed to involvement of integrin receptors $\alpha v\beta 3$, $\alpha 5\beta 1$ or $\alpha IIb\beta 3$, this may not always be so. Eristostatin, from *Eristocophis macmahoni*, is a potent inhibitor of ADP-induced platelet aggregation as well as of human and murine melanoma metastases in mouse model systems. This disintegrin requires an RGDW motif, as well as an intact C-terminus, in order to interact with both platelets and four different types of melano-

ma cells. Eristostatin causes nonmetastatic SBc12 melanoma cells to show higher susceptibility to specific killing by NK-like TALL-104 cells. While it is known that eristostatin binds to $\alpha IIb\beta 3$ on platelets, the receptor with which eristostatin binds to the melanoma cells has not yet been identified.

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Introduction

Disintegrins are low molecular weight, cysteine-rich proteins isolated from viper venom [1]. Previous investigations have demonstrated that these proteins interact with many types of cells, such as platelets [2], neutrophils [3], human umbilical vein [4] and bovine capillary [5] endothelial cells, and many types of cancer cells, including breast [6], neuroglial [7], bladder [8] and melanoma [9]. Whenever

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0301-0147/01/0316-0177\$17.50/0

Accessible online at:
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the investigations have attempted to identify the receptor through which the interaction is achieved, an integrin is usually involved. This is understandable since all disintegrins possess binding motifs (RGD, KGD, MVD, MLD, VGD) similar to those found in native ligands, such as fibronectin, fibrinogen and VCAM-1 [10–12]. Disintegrins, as a family of proteins, simultaneously share significant sequence homology and cysteine arrangement, yet demonstrate substantial diversity in their binding interactions with cellular integrins. We previously demonstrated that certain non-RGD motifs present in these adhesive ligands will also contribute to their binding selectivity for α IIB β 3, α v β 3 and α 5 β 1 integrins [13]. We have used a panel of recombinant mutants of eristostatin (based on the native protein from *Eristocophis macmahoni*) to investigate whether the binding motifs required for interacting with ‘normal’ cells are also required with transformed, cancerous cells. We also hypothesize a mechanism for the ability of eristostatin to inhibit melanoma cell metastasis.

Material and Methods

Chemicals and Media

Standard laboratory chemicals were obtained from Sigma (St. Louis Mo., USA). Eight-chambered cover-slip slides were from Nalge Nunc (Naperville, Ill., USA). Dulbecco's media and PBS were obtained from Mediatech (Herndon, Va., USA). Fetal calf serum was from GibcoBRL (Rockville Md., USA). $H_3^{51}CrO_4$ was obtained from Perkin-Elmer (Boston, Mass., USA). Antibodies against α 4 integrin subunit were purchased from Immunotech (Marseille, France) and antibodies against CD48, CD27, CD70, CD137, CD137-ligand, CD134, and FAS were purchased by Pharmingen (San Diego, Calif., USA). For flow cytometry analysis, fluoresceinated secondary antibodies were purchased from Organon Technika Corporation (Durham, N.C., USA).

Cells Used

Human melanoma cell lines 1205-LU, WM164 and SBc12 were kindly provided by Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, Pa., USA), while C8161 and MV3 cells were obtained from Danny Welch (Penn State University, Hershey, Pa., USA) and Stefan Niewiarowski, (Temple University, Philadelphia, Pa., USA), respectively. Cells were cultured in Dulbecco's Modified Eagle's medium/F12 containing L-glutamine and supplemented with 10% fetal calf serum (FCS). Cells were grown at 37°C, 5% CO₂, detached using 2 mmol/l EDTA and resuspended in Dulbecco's phosphate-buffered saline. Cytotoxic cell line TALL-104 (MHC-nonrestricted, CD8+, CD56+, CD16-, CD161+) were grown in Iscove's Modified Dulbecco's Medium, supplemented and grown as above.

Preparation of Disintegrins

Wild-type eristostatin and five mutations listed in table 1 were expressed in *Escherichia coli* as described previously [13]. ADP-induced platelet aggregation was used to screen each disintegrin, and the IC₅₀ values obtained agreed with those previously published [13]. FITC-labeling of eristostatin was performed using a Fluorotag conjugation kit from Sigma, following the manufacturer's directions. The ratio of fluorescence to protein of the labeled disintegrin was 1:6.

Binding Inhibition

Each human melanoma cell line (3E5) was incubated with buffer or unlabeled disintegrin (500–3,000 nM). Without washing, the cells were then stained with FITC-labeled eristostatin (for 1205-LU, WM164, C8161) or FITC-labeled anti- α 4 (for MV3). All data were acquired on a Zeiss inverted 100M Axioskop equipped with a Zeiss 510 LSM confocal microscope and a krypton-argon laser. Each binding pattern was rated from zero (no inhibition) to +3 (complete inhibition), depending on how much the disintegrin inhibited the binding of the FITC-labeled protein.

Flow Cytometry Analysis

SBc12 melanoma cells were incubated for 3 days in the presence or absence of 500 nM eristostatin. After washing, the cells were stained with primary antibodies to cell surface markers, washed and probed with FITC-conjugated secondary antibody. Flow analysis was performed on an Epics XL flow cytometer, and nonspecific fluorescence (using 1 mg FITC-albumin) showed less than 0.5 fluorescence units.

Table 1. Mutations made in RGD loop and C-terminus of eristostatin or echistatin

Forms of eristostatin	Location of mutation within sequence (compared with wildtype eristostatin)
Eristostatin (wildtype)	²³ CRVARGDWNDDYCTGKSCDCPRNPWNG ⁴⁹
Eristo R27A	A
EristoW30D	D
Eristo N31M	M
Eristo W30D/N31M	DM
EristoV25R/W30D	R D
Eristo (1-43)	RNPWNG

For each mutation, the amino acid substitution is placed under its corresponding wildtype residue. Those motifs which are removed are indicated by a double crossout.

Cell Cytotoxicity

Sensitivity of human melanoma SBc12 cells, incubated overnight with 500 nM eristostatin or media control, to the cytotoxic action of TALL-104 cells was tested in an 18 h ⁵¹Cr release assay, performed as previously described [14]. Briefly, SBc12 target cells were resuspended at 1E6/ml in PBS and labeled for 1 h at 37°C with 0.1 mCi of H₃⁵¹CrO₅. After washing, cells were resuspended at 1E4/50 µl and incubated at 37°C in 10% CO₂ with serially diluted effector TALL-104 cells. After 18 h, cell supernatant was collected, and released radioactivity was measured with a gamma counter. Percent of specific radioactivity was calculated as: sample radioactivity – spontaneous release/total radioactivity (Triton-lysed cells) – spontaneous release × 100.

Results and Discussion

Eristostatin is unique among the venom-derived disintegrins for a number of reasons. It is a potent inhibitor of ADP-induced platelet aggregation (IC₅₀ = 70 nM), and binds αIIbβ3 integrin with a K_d of 18 nM, whether the platelets are resting or activated by ADP or thrombin [2]. It has been shown to inhibit lymphocyte infiltration of pancreatic islets in nonobese diabetic mice [Clara Brando, unpubl. obs.], and is able to inhibit melanoma

metastasis in an in vivo model of experimental metastasis, using either murine [15, 16] or human [9] melanoma cells. It shares an integrin selectivity property with another disintegrin, barbourin [17], since it has been shown to interact with cells primarily through the integrin αIIbβ3 [2], although Danen et al. [9] did demonstrate a possible binding to α4β1 on MV3 melanoma cells. In contrast to most other disintegrins, however, eristostatin does not interact with αvβ3 or α5β1 [13]. It is curious that the murine melanoma cells used in the experimental metastasis assays (BL16F10, BL16F1) showed a strong expression of β3, but the human MV3 cells did not. This would suggest that eristostatin may not use αIIbβ3 consistently, if at all, in its interactions with the melanoma cells whose metastasis it inhibits. As the first step in determining the molecular mechanism used by eristostatin to inhibit human melanoma metastasis, we used our panel of recombinant eristostatin mutants (table 1) to demonstrate which motifs within eristostatin are most critical for the in vitro interaction of eristostatin with four human melanoma cell lines (MV3, C8161, 1205-LU, WM164) by confocal microscopy

Table 2. Summary of all cell lines for inhibition of binding by recombinant disintegrins

Disintegrin	1205-LU	WM164	C8161	MV3
Eristostatin	+2	+3	+3	+3
ErR27A	0	0	0	0
ErW30D	+2	+2	+2	0
ErV25R/W30D	+2	+2	+2	+1
ErW30D/N31M	+3	+3	+3	0
ErN31M	+3	+3	+3	0
Eristostatin(truncated)	0	0	0	0

Each human melanoma cell line (3E5) was incubated with buffer or the indicated disintegrin (500–3000 nM). Without washing, the cells were then stained with FITC-labeled eristostatin (for 1205-LU, WM164, C8161) or FITC-labeled anti- α 4 (for MV3), and observed by confocal microscopy. Each binding pattern was rated from 0 (no inhibition) to +3 (complete inhibition), depending on how much the disintegrin inhibited the binding of the FITC-labeled protein. The data are representative of a minimum of 2 identical experiments.

(table 2). The four melanoma cell lines were used to determine whether the interaction of eristostatin with human melanoma cells was unique to MV3. In fact, eristostatin bound to all four cell types. This was directly shown with 1205-LU, WM164 and C8161 cells through confocal microscopy using FITC-eristostatin. For MV3 cells, we demonstrated the interaction of eristostatin by showing a dose-dependent inhibition of the binding of anti- α 4, confirming the findings of Danen et al. [9] that eristostatin interacts with α 4 β 1 on these cells. As summarized in table 2, binding to 1205-LU, WM164, and C8161 melanoma cells requires an **RGD** sequence and an intact C-terminus. Asparagine is not needed in the RGD loop of eristostatin for binding to these cells. Surprisingly, methionine at position **RGDXX** enhances the ability of eristostatin to bind. For MV3 melanoma cells, an **RGD** sequence, an intact C-terminus, and both asparagine and tryptophan in the RGD loop of eristostatin are required. We hypothesize that eristostatin uses the same molecular mecha-

nism to bind to 1205-LU, WM164, and C8161 melanoma cells. However, the melanoma cell receptor by which eristostatin accomplishes this interaction is as yet unidentified. Based on the pattern of eristostatin mutation results, we propose that the mechanism at work in its interactions with MV3 cells is different from the one used in binding to 1205-LU, WM164, and C8161 cells.

In order to study the consequences of the binding of eristostatin to melanoma cells, we first incubated SBc12 human melanoma cells $\pm$ 4000 nM eristostatin for 5 days, and observed no difference between the control and treated cells in cell count or gross appearance, confirming the findings of Danen et al. [9] and Morris et al. [16] (data not shown). We then tested whether the presence of eristostatin would make a difference in the ability of cytotoxic cells to kill the SBc12 melanoma cells. As shown in figure 1, TALL-104 cytotoxic cells [18] are capable of killing the SBc12 melanoma cells, in a dose-dependent fashion, without the presence of eristostatin. However,

Fig. 1. Human melanoma SBcl2 cells were incubated in the presence or absence of eristostatin for 3 days at 37°C. After washing, the cells were counted and 10^6 cells were suspended in media to which was added 100 μCi $\text{H}_3^{51}\text{CrO}_4$ for 1 h. After washing with cold, complete media, these 'target' cells were distributed in 96-well plates, incubated with serial dilutions of effector cells: MHC-nonrestricted TALL-104 cells (CD8+, CD56+, CD16-, CD161+), which had been similarly incubated with or without eristostatin. After overnight incubation, the supernatant was harvested and the

released radioactivity was counted with a gamma counter. Calculation of percent killing was expressed as: $\frac{\text{sample radioactivity} - \text{spontaneous release}}{\text{total radioactivity (Triton-lysed cells)} - \text{spontaneous release}} \times 100$. Data shown are representative of three identical experiments. ● = Untreated cytotoxic cell/melanoma cell; ▲ = treated cytotoxic cell, untreated melanoma cell; ○ = treated cytotoxic cell, treated melanoma cell; ■ = untreated cytotoxic cell, treated melanoma cell.

when the melanoma cells are incubated with eristostatin, TALL-104 cells show a 2-fold increased cytotoxic effect. One possible explanation is that incubation with eristostatin increases the expression of receptors for cytotoxic substances, secreted by effector cells, on the membrane of the target cell. Thus SBcl2 were screened for the presence of Fas, CD27, CD134, CD137, and CD137-L by flow analysis. No differences were found between eristostatin-treated and control SBcl2 cells.

Another explanation could be that costimulatory molecules for the process of killing are modulated by eristostatin. Thus, cells were analyzed for the presence of CD48,

ligand for 2B4, a costimulatory molecule expressed by cytotoxic cells [19]. Expression of CD48 is not affected by treatment with eristostatin. Studies are under way to identify the molecule with which eristostatin binds on these melanoma cells, and the intracellular consequences of this interaction.

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