Pro-apoptotic signaling pathway activated by echistatin in GD25 cells

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Abstract

Disintegrins, low molecular weight RGD-containing polypeptides isolated from snake venoms, have seen use as integrin antagonists in the field of tumor biology and angiogenesis. In this study, we investigated the molecular mechanism by which the disintegrin echistatin affects cell adhesion and signaling resulting in an apoptotic response in the GD25 cell system. Wild-type GD25 cells, which lack expression of the $\alpha_1$ family of integrin, and stable transfectants expressing the A isoform of $\alpha_1$ integrin subunit were used.

Nanomolar concentrations of echistatin detached fibronectin- and vitronectin-adherent GD25 cells from immobilized substratum. However, prior to inducing detachment of adherent cells, echistatin caused apoptosis as measured by caspase-3 activation. Either cell detachment or apoptotic response induced by echistatin were more pronounced on fibronectin-adherent GD25 cells than on vitronectin-adherent ones.

GD25 cell exposure to echistatin caused a reduction of tyrosine phosphorylation levels of pp125FAK, whereas it didn’t affect either Shc tyrosine phosphorylation levels or Shc–Grb2 functional association. The down-regulation of pp125 FAK preceded apoptosis and cell detachment induced by echistatin. Our results indicate that pp125 FAK and not Shc pathway is involved in echistatin-induced apoptotic response in the GD25 cell system.

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1. Introduction

Disintegrins are a family of low molecular weight proteins isolated from Crotalidae and Vipiridae snake venoms [1]. They contain an Arg–Gly–Asp (RGD) motif which represents a common adhesion recognition site [2]. However, disintegrins with other recognition motifs such as KGD, VGD or MLD have been identified [3–5]. Disintegrins bind with high affinity integrin receptors on platelet and cell surface, thus inhibiting both platelet aggregation and cell adhesion [6]. Additional structural features other than the RGD motif, including the amino acids flanking the RGD sequence, the pattern of intramolecular S=S bridges, and the presence in their molecule of other recognition sites affect disintegrin activity [7]. Thus, structurally distinct disintegrins may differently act on cell adhesion and signaling [8,9].

Disintegrins have seen use as integrin antagonists in the fields of tumor biology and angiogenesis [10,11]. Various disintegrins have been shown to inhibit angiogenesis either in vitro and in vivo by inducing apoptosis [12]. Echistatin was shown to induce an apoptotic response occurring prior to cell detachment in $\alpha_5\beta_3$-transfected human embryonic kidney-293 epithelial cells [13]. However, the molecular mechanism of action of disintegrins in causing apoptosis has not been fully clarified yet.

We previously showed that echistatin inhibits experimental Lewis lung carcinoma-induced metastasis in vivo [14] and we demonstrated that this disintegrin detaches fibronectin-adherent melanoma cells by down-regulating pp125 FAK phosphorylation [15]. The echistatin-induced dephosphorylation of pp125 FAK Tyr 118 residue resulted in a decrease of paxillin tyrosine phosphorylation and in the disruption of pp125 FAK–Src and pp125 FAK–paxillin func-
tional complexes [16]. Herein, we also demonstrated that a reduction of tyrosine phosphorylation of pp125FAK, paxillin and Shc is involved in apoptosis of COS cells induced by ochratoxin A, a mycotoxin with nephrotoxic, teratogenic and carcinogenic activity which seems to be involved in the pathogenesis of Balkan Endemic Nephropathy and urinary tract tumors [17].

In this study, we evaluated the ability of echistatin to affect cell adhesion and signaling in the mouse cell line GD25. These cells, derived from the embryonic stem cell clone G201, lack expression of the β1 family of integrin heterodimers due to the disruption of the β1 subunit gene [18]. By using wild-type GD25 cells and stable transfectants of these cells expressing β1A isoform of β1 integrin subunit, the molecular mechanism by which echistatin induces an apoptotic response was investigated. In particular, the FAK and Shc signaling pathways activated by the disintegrin interaction with integrin receptor(s) were characterized.

2. Materials and methods

2.1. Antibodies

Hors eradish peroxidase conjugated goat anti-(mouse IgG) Ig and hors eradish peroxidase conjugated goat anti-(rabbit IgG) Ig were purchased from Sigma Chemical Co. (St. Louis, MO, USA); rabbit polyclonal anti-human FAK IgG from Upstate Biotechnology (Lake Placid, NY, USA); monoclonal anti-phosphotyrosine (PY20) IgG, monoclonal mouse anti-Grb2 IgG and rabbit polyclonal anti-Shc Ig from Transduction Laboratories (Lexington, KY, USA).

2.2. Chemicals

Aprotinin, bovine serum albumin (BSA), Dulbecco minimum Eagle’s medium (DMEM), human plasma fibronectin, leupeptin, orthovanadate, pepstatin, N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC), 1,4-piperazinediethane sulfonic acid (PIPES), 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (CHAPS), ethylenediaminetetraacetic acid (EDTA), 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (DDT) were purchased from Sigma Chemical Co.; l-glutamate and trypsin from ICN Biomedicals, Inc. (Aurora, OH, USA); fetal bovine serum (FBS) from Hyclone Laboratories, Inc. (Logan, UT, USA); protein A-agarose and protein G-agarose from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.3. Cell culture

GD25 cells were kindly provided by Prof. Francesco Retta (Dipartimento di Genetica, Biologia e Biochimica, Università di Torino, Torino, Italy). GD25 cells were grown on plastic Petri dishes in DMEM supplemented with 10% FBS and 1% l-glutamine at pH 7.4, and cultured under standard cell culture conditions (37 °C, 5% CO2). In the culture medium of GD25 β1A cells hygromycin B was also added. Cells were harvested for propagation or cell attachment studies by treatment with 0.25% trypsin/0.02% EDTA in phosphate-buffered saline (PBS), pH 7.2, and with 5 mM EDTA in PBS, respectively. Cells were then washed with DMEM and resuspended in complete DMEM for propagation or in DMEM with 2% FBS for adhesion experiments.

2.4. Substrate coating

Tissue culture plastic dishes (35 × 10 mm) or 96-multiwell plastic dishes (Costar, Cambridge, MA, USA) were coated overnight at 4 °C by incubation with human plasma fibronectin (10 μg/ml) diluted in PBS with 1 mM CaCl2 and 1 mM MgCl2, or with vitronectin (5 μg/ml) diluted in PBS. After coating, dishes were treated with 1% BSA in PBS for 30 min at 37 °C to block free binding sites on the plastic. Before plating cells, dishes were rinsed twice with PBS.

2.5. Cell adhesion assay

Freshly suspended cells (100 μl, 75 × 104 cells/ml) were plated onto 96-multiwell plates previously coated with fibronectin or vitronectin and allowed to adhere for 3 h at 37 °C. Non-adherent cells were removed by gentle washing with PBS. Adherent cells were exposed to 5 μg/ml of echistatin for increasing time intervals (0–6 h) or to different concentrations of echistatin (0.1–20 μg/ml) for 3 h and incubated at 37 °C. At the indicated time, detached cells were gently removed by washing with PBS, adherent cells were fixed, and stained by crystal violet. The colour yields were measured by a Bio-Rad ELISA reader equipped with a 570 nm filter. The percentage of cell detachment induced by echistatin was evaluated as previously described [15].

2.6. Apoptosis assay

Freshly suspended cells (1 × 106 cells) were plated on substratum-coated dishes, allowed to adhere at 37 °C for 3 h. Non-adherent cells were removed by gentle washing with serum-free medium. Adherent cells were exposed to increasing amounts of echistatin (0.1–10 μg/ml) for 3 h or to 5 μg/ml of echistatin for increasing time intervals (0–180 min). At the end of the incubation time, cells were lysed by ice-cold lysis buffer containing 10 mM Tris—HCl, pH 7.5, 125 mM NaCl, 1% Triton, 10 mM Na2HPO4, 22.7 μg/ml aprotinin (0.1 trypsin inhibitor units/ml), 10 μg/ml leupeptin, 0.4 mM Na3VO4. The lysates were clarified by centrifugation at 12,000 × g for 10 min at 4 °C. The amount of proteins in the samples was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories). Lysates containing 150 μg of proteins were incubated with 50 μM of Ac-DEVD-AMC in 1 ml of reaction buffer (10 mM PIPES,
pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT) for 5 h at 37 °C. Fluorescence derived from the release of the 7-amino-4-methylcoumarin moiety was followed using a PerkinElmer with λ excitation at 390 nm and λ emission at 460 nm. The data obtained are reported as the variation (Δ) of the fluorescence of the treated sample lysate minus the fluorescence of the appropriate control sample lysate.

2.7. Protein immunoprecipitation and Western blotting

Freshly suspended cells (1 × 10^6 cells) were plated on substrate-coated dishes and allowed to adhere at 37 °C for 3 h. Non-adherent cells were removed by two gentle washings with serum-free medium, adherent cells were exposed to 20 μg/ml of echistatin for 1 or 3 h. At the end of the incubation time, the proteins were detergent-extracted by ice-cold lysis buffer, as described in Apoptosis assay. The lysates were clarified by centrifugation at 12,000 × g for 10 min at 4 °C and the amount of proteins in the samples was determined by the Bio-Rad DC protein assay.

Lysates containing equal amount of proteins were incubated with anti-pp125^Fak (1 μg 100 μg proteins) or anti-Shc Ig (0.25 μg 100 μg proteins) for 6 h at 4 °C. Protein A- or protein G-agarose was then added to the samples and incubated overnight at 4°C. Beads were sedimented by brief centrifugation and immunoprecipitates were washed extensively with cold lysis buffer. Proteins were resuspended in SDS sample buffer, boiled for 5 min in Laemmli buffer and run on 10% SDS/polyacrylamide gel.

After electrophoresis, the proteins were transferred to nitrocellulose using a Mini trans-blot apparatus (Bio-Rad Laboratories) according to manufacturer’s instructions. Membranes were blocked for 1 h at 42 °C with blocking buffer containing 5% BSA in Tris/NaCl/Pi and incubated overnight with a 1:2000 dilution of antibody PY20 to probe for phosphotyrosine-containing proteins. The blots were washed three times with Tris/NaCl/Pi/Tween (150 mM NaCl, 20 mM Tris–HCl, pH 7.4, 0.3% Tween 20) and incubated for 1 h with peroxidase conjugated anti-(mouse IgG) Ig diluted 1:3000 in Tris/NaCl/Pi, 1% BSA.

To evaluate the amount of Grb2 co-precipitating with Shc, cellular extracts were immunoprecipitated with anti-Shc Ig as previously described, and separated by SDS gel electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose using a Mini trans-blot apparatus (Bio-Rad Laboratories) according to manufacturer’s instructions. The membranes were blocked for 1 h with blocking buffer containing 5% milk in Tris/NaCl/Pi and incubated overnight with a 1:500 dilution of antibody Grb2. The blots were washed three times with Tris/NaCl/Pi/Tween (150 mM NaCl, 20 mM Tris–HCl, pH 7.4, 0.3% Tween 20) and incubated for 1 h with peroxidase conjugated anti-(rabbit IgG) Ig diluted 1:3000 in Tris/NaCl/Pi, 2.5% milk.

The proteins were visualized by an ECL chemiluminescence kit (Amersham Corp., Little Chalfont, UK). The same blots were stripped and reprobed using anti-pp125^Fak or anti-Shc Ig to confirm equal loading of pp125^Fak or Shc in the lysates.

3. Results

3.1. Echistatin causes detachment of GD25 cells from immobilized fibronectin or vitronectin

The ability of echistatin to promote cell detachment from immobilized substrates was evaluated. Echistatin induced GD25 cell detachment from immobilized fibronectin, but this effect was more pronounced on fibronectin-adherent GD25 β1A cells than on wild-type GD25 cells. Cell treatment with 20 μg/ml of echistatin for 3 h induced 87% of GD25 β1A cell detachment from immobilized fibronectin, while the same dose caused 76% of wild-type GD25 cell detachment from immobilized fibronectin (Fig. 1, panel A). Echistatin promoted cell detachment from immobilized vitronectin, but this effect was more pronounced on wild-type GD25 cells than on GD25 β1A: 3 h of cell treatment with 20 μg/ml echistatin caused 45% and 54% of cell detachment for GD25 β1A cells and wild-type GD25 cells, respectively (Fig. 1, panel A).

The time-course of echistatin-induced cell detachment confirmed the observation that the disintegrin is more active to promote cell detachment from fibronectin than from vitronectin and that wild-type cells are easier detached from vitronectin than GD25 β1A cells, whereas GD25 β1A are more easily detached from immobilized fibronectin than wild-type cells (Fig. 1, panel B).

We had previously observed that the attachment to immobilized fibronectin and vitronectin which bind α5β1 and αvβ3 integrin receptors on cell surface, respectively, is efficient for both wild-type GD25 cells and the GD25 β1A cells. Furthermore, β1A transfected GD25 cells showed a higher percentage of adhesion to immobilized fibronectin than wild-type GD25 cells, whereas the percentage of wild-type GD25 cells adhering to immobilized vitronectin was higher than that of transfected-ones [19]. This may be due to the observed overexpression of the αvβ3 integrin receptor, specific for vitronectin, on wild-type cells which lack the β1 integrin subunit [20]. Thus, the results obtained indicate that echistatin is able to promote cell detachment by competing with both immobilized fibronectin and vitronectin for their respective integrin receptor. However, echistatin activity was more pronounced on fibronectin-adherent GD25 cells than on vitronectin-adherent GD25 cells, thus suggesting that the disintegrin has higher affinity for fibronectin receptor than for vitronectin receptor.

3.2. Echistatin promotes apoptosis of GD25 cells adhering to immobilized fibronectin or vitronectin

The ability of echistatin to promote apoptosis in adherent GD25 cells was evaluated by measuring the activation of
caspase-3, which represents an early hallmark during apoptosis [21]. Echistatin caused apoptosis in both wild-type GD25 and GD25 b1A cells cultured on either immobilized vitronectin or fibronectin. Fig. 2 reports the increase in the fluorescence as measured in echistatin-treated cells with respect to control (untreated) cells.

Echistatin-induced apoptotic response was more pronounced on fibronectin-adherent GD25 b1A cells than on vitronectin-adherent ones (Fig. 2, panel A).

Interestingly, a significant increase in the fluorescence which is strictly correlated to the activation of caspase-3 was observed at echistatin doses lower than those causing cell detachment and at an earlier time than that required to induce cell detachment.

### 3.3. Echistatin activity on tyrosine phosphorylation and functional association of focal adhesion associated-proteins

The ability of echistatin to modulate tyrosine phosphorylation levels of the focal adhesion-associated proteins FAK and Shc was evaluated. A reduction of tyrosine phosphory-
lation levels of pp125FAK was observed in protein lysates from wild-type GD25 and GD25 β1A cells cultured either on immobilized fibronectin or vitronectin and exposed to echistatin (Fig. 3, panels A and B, upper blots). To confirm equal loading of pp125FAK in all lanes, the blots were stripped and reprobed with an anti-FAK antibody (Fig. 3, panels A and B, lower blots). The cells were exposed to the same dose of echistatin for 3 h (data not shown); however, the results obtained are consistent with the ones obtained after 1 h of cell treatment.

Next, the effect of echistatin treatment of adherent wild-type GD25 and GD25 β1A cells on Shc tyrosine phosphorylation was observed in protein lysates from wild-type GD25 and GD25 β1A cells cultured either on immobilized fibronectin or vitronectin and exposed to echistatin (Fig. 4, panels A and B, upper blots). The cells were exposed to the same dose of echistatin for 3 h (data not shown); however, the results obtained are consistent with the ones obtained after 1 h of cell treatment.

Fig. 3. Echistatin activity on tyrosine phosphorylation of pp125FAK in adherent cells. Wild-type GD25 (lanes 1 and 2) and GD25 β1A cells (lanes 3 and 4) were allowed to adhere to immobilized fibronectin (panel A) or vitronectin (panel B) for 3 h and then exposed to 20 μg/ml of echistatin for 1 h (upper blots, lanes 2 and 4). Control cells were treated with fresh culture medium for 1 h (upper blots, lanes 1 and 3). Cell lysates were immunoprecipitated with an anti-FAK Ig and subsequently separated by electrophoresis. After Western blotting, tyrosine phosphorylated proteins were visualized by an anti-phosphotyrosine Ig. The same membranes used in the upper blots of panels A and B were stripped and reprobed with an anti-FAK Ig to ensure that the same amount of pp125FAK was immunoprecipitated from all samples (lower blots). Molecular mass markers are indicated on the left. The position of pp125FAK is indicated by the arrows on the left. Similar results were obtained from three separate experiments of identical design.

Fig. 4. Echistatin activity on tyrosine phosphorylation of Shc in adherent cells. Wild-type GD25 (lanes 1 and 2) and GD25 β1A cell (lanes 3 and 4) were allowed to adhere to immobilized fibronectin (panel A) or vitronectin (panel B) for 3 h and then exposed to 20 μg/ml of echistatin for 1 h (upper blots, lanes 2 and 4). Control cells were treated with fresh culture medium for 1 h (upper blots, lanes 1 and 3). Cell lysates were immunoprecipitated with an anti-Shc Ig, and subsequently separated by electrophoresis. After Western blotting, tyrosine phosphorylated proteins were visualized by an anti-phosphotyrosine Ig. The same membranes used in upper blots of panels A and B were stripped and reprobed with an anti-Shc Ig to ensure that the same amount of Shc was immunoprecipitated from all samples (lower blots). Molecular mass markers are indicated on the left. The position of Shc isoforms are indicated by the arrows on the left. Similar results were obtained from three separate experiments of identical design.
ulation levels was investigated. Only the p52 isoform of Shc appears to be phosphorylated in wild-type GD25 and GD25 β1A cells adherent to immobilized substrata. A decrease of tyrosine phosphorylation of p52 Shc isoform was only observed in vitronectin-adherent GD25 β1A cells exposed to echistatin (Fig. 4, panel B, upper blot), whereas the disintegrin did not modify the tyrosine phosphorylation levels of Shc protein of both fibronectin- and in vitronectin-adherent wild-type GD25 and GD25 β1A cells (Fig. 4, panels A and B, upper blots). In order to evaluate whether the Shc loading was equal in all lanes, the blots were stripped and reprobed with an anti-Shc antibody (Fig. 4, panels A and B, lower blots).

Finally, the action of echistatin on the functional association of Shc with Grb2 was evaluated. Shc immune complexes prepared from lysates of substrata-adherent wild-type GD25 and GD25 β1A cells were probed with anti-Grb2 Ig in order to determine the presence of co-precipitating Grb2. The association of Grb2 with Shc was observed in wild-type GD25 and GD25 β1A cells held in suspension (data not shown) or adherent to both substrata. The addition of echistatin to vitronectin- and fibronectin-adherent GD25 cells did not cause a reduction of the amount of Grb2 co-precipitating with Shc in both wild-type GD25 and GD25 β1A cells (Fig. 5, panel A and B, upper blots). The same blots were stripped and reprobed with an anti-Shc antibody to ensure that the loading of the protein was equal in all lanes (Fig. 5, panels A and B, lower blots).

4. Discussion

The β1 integrin subunit is expressed in most mammalian cells except mature erythrocytes [22]. Three splice variants of the protein have been described and some cells express more than one variant at the same time [23]. The different forms of the protein have specific functions, as indicated by the inability of β1B isoform to promote cell migration, to activate pp125FAK or to localize to focal contacts [20].

Echistatin, a 49 amino acid disintegrin found in the venom of *Echis carinatus*, is a potent inhibitor of ligand binding to αIIbβ3, αvβ3 and α5β3 [24]. The disintegrin uses different structural motifs, including the RGD loop and C terminus, to selectively interact with α5β1 and αvβ3 receptors [25]. Here, we show that echistatin is able to detach GD25 cells from either immobilized fibronectin or vitronectin. The activity of disintegrin was specific, not due to toxicity, and was dependent on the dose and the time of cell exposure. In particular, echistatin detached the GD25 β1A cells from fibronectin at a greater extent than wild-type GD25 cells. Conversely, the disintegrin detached the wild-type GD25 cells from vitronectin at a greater extent than GD25 β1A cells (Fig. 1). Our results suggest that echistatin binds with higher affinity the integrin α5β1 than integrin αvβ3; this finding is in agreement with the observation of a lower binding affinity of echistatin for αvβ3 and of the dissociable nature of the disintegrin interaction with αvβ3 receptor [26].

![Fig. 5. Echistatin activity on functional association of Shc with Grb2 in adherent cells.](image)

Wild-type GD25 (lanes 1 and 2) and GD25 β1A cell (lanes 3 and 4) were allowed to adhere to immobilized fibronectin (panel A) or vitronectin (panel B) for 3 h and then exposed to 20 μg/ml of echistatin for 1 h (upper blots, lanes 2 and 4). Control cells were treated with fresh culture medium for 1 h (upper blots, lanes 1 and 3). Cell lysates were immunoprecipitated with an anti-Shc Ig, and subsequently separated by electrophoresis. After Western blotting, the association of the proteins was visualized by an anti-Grb2 Ig. The same membranes used in upper blots of panels A and B were stripped and reprobed with an anti-Shc Ig to ensure that the same amount of Shc was immunoprecipitated from all samples (lower blots). Molecular mass markers are indicated on the left. The position Grb2 is indicated by the arrows on the left. Similar results were obtained from three separate experiments of identical design.
Echistatin was able to promote apoptosis in both types of GD25 cells cultured on immobilized substrata. The effect of echistatin was more pronounced on GD25 \( \beta_1 \)A cells than on wild-type GD25 cells adhering to fibronectin and more pronounced on wild-type GD25 cells than on GD25 \( \beta_1 \)A cells adhering to vitronectin (Fig. 2). Furthermore, the apoptotic response induced by echistatin in the GD25 cells occurred prior to cell detachment. Cell substratum detachment usually induces an apoptotic response (anoikis); however, echistatin binding to \( \alpha_\beta_3 \) integrin has been shown to induce cell death resulting in an apoptotic signal with different kinetics than the apoptotic signal induced by matrix detachment [13]. This apoptotic inducing effect of echistatin may be due to the ability of disintegrin to cause integrin-mediated death (IMD) [23]. This form of apoptosis is biologically and biochemically distinct from anoikis, which is due to cell detachment from substratum. Herein, IMD is defined as the apoptosis of adherent cells induced by the recruitment of caspase-8 to the \( \beta \) subunit tails of unligated integrins. This is a cellular process whereby adherent cells that find themselves within an inappropriate extracellular matrix (into the tissues), or those exposed to integrin antagonists would be actively cued to undergo apoptosis. Interestingly, while \( \beta_1 \) and \( \beta_3 \) integrins can promote this process, \( \beta_5 \) does not [27]. Echistatin may cause IMD on GD25 cells adhering to vitronectin and fibronectin by binding \( \alpha_\beta_3 \) and \( \alpha_5\beta_1 \) integrins.

Because of the complexity of signaling pathways involved in apoptotic response by chemicals and because of the cell-type specificity of signaling activation that leads to apoptosis, whether FAK and Shc pathways are involved in the apoptotic signals induced by echistatin was investigated. Thus, the ability of echistatin to modulate tyrosine phosphorylation levels of the two proteins was evaluated.

Echistatin treatment caused a reduction of tyrosine phosphorylation of pp125\(^{\text{FAK}}\) in protein lysates from GD25 cells immobilized either on fibronectin or vitronectin (Fig. 3). No differences were observed in the signaling pathway activated by echistatin treatment of either wild-type GD25 cells or transfected-ones. However, integrin signaling independent of ligand recognition has been described [27].

FAK is a crucial molecule in integrating signals from integrins and receptor tyrosine kinases in processes such as cell survival, proliferation and motility [28]. Herein, FAK phosphorylation levels are closely associated with the stability of focal adhesions and cell–matrix interactions. During cell adhesion process, turnover of focal adhesions requires activation of FAK and its functional interactions with several proteins involved in integrin signaling [29]. For example, FAK-deficient cells spread more slowly on extracellular matrix proteins exhibiting an increased number of prominent focal adhesions; these cells migrate poorly in response to chemotactic signals [30]. Furthermore, cells lacking paxillin expression, Cas expression or Src family kinase expression all exhibit defects in focal adhesion turnover [31]. The disassembly of focal adhesions and the subsequent GD25 cell detachment from the substratum induced by echistatin seem to be later events than focal adhesion protein dephosphorylation. Although disruption of focal adhesion signaling through FAK clearly precedes cell detachment after echistatin induced apoptosis, our data also suggest that caspase-3 activation is not responsible for the former. Our results suggest that the dephosphorylation of FAK may contribute to the loss of focal adhesions and cell detachment.

Using human breast cancer cell lines, Xu et al. [32] showed that FAK signaling pathway is involved in inhibiting death receptor or death receptor-related apoptosis. The overexpression in the cells of the carboxyl-terminal domain of FAK, that is without kinase activity, induced apoptosis independently of cell adhesion, but required Fas-associating protein with death domain (FADD), caspase-8 and caspase-3 [32].

The adapter protein Shc has also been linked to cytoskeletal organization of cells under different contexts [33]. Some reports suggest distinct roles for Shc and FAK in focal adhesions and cell motility [34,35]. However, how Shc plays a role in the cytoskeletal reorganization in different cell types is not well understood [36]. GD25 cell exposure to echistatin does not appear to affect either tyrosine phosphorylation levels of Shc isoforms (Fig. 4) or the functional association Shc–Grb2 (Fig. 5).

Our results indicate that FAK pathway, but not Shc pathway is involved in the pro-apoptotic signals induced by echistatin in GD25 cell system, during integrin-mediated death process. However, further studies are needed in order to identify FAK downstream molecules involved in the echistatin-induced apoptotic response. These studies aimed at a better understanding of the molecular mechanism of action of disintegrins may open the way to therapeutic targets for human diseases which require a modulation of integrin function.

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