Inhibition of integrin-mediated adhesion and signaling disrupts retinal development

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Abstract

Integrins are the major family of cell adhesion receptors that mediate cell adhesion to the extracellular matrix (ECM). Integrin-mediated adhesion and signaling play essential roles in neural development. In this study, we have used echistatin, an RGD-containing short monomeric disintegrin, to investigate the role of integrin-mediated adhesion and signaling during retinal development in Xenopus. Application of echistatin to Xenopus retinal-derived XR1 glial cells inhibited the three stages of integrin-mediated adhesion: cell attachment, cell spreading, and formation of focal adhesions and stress fibers. XR1 cell attachment and spreading increased tyrosine phosphorylation of paxillin, a focal adhesion associated protein, while echistatin significantly decreased phosphorylation levels of paxillin. Application of echistatin or \( \beta_1 \) integrin function blocking antibody to the embryonic Xenopus retina disrupted retinal lamination and produced rosette structures with ectopic photoreceptors in the outer retina. These results indicate that integrin-mediated cell–ECM interactions play a critical role in cell adhesion, migration, and morphogenesis during vertebrate retinal development.

Keywords: Integrin; Focal adhesion; Disintegrin; Echistatin; Retinal development

Introduction

Cells in the developing retina contact a variety of molecular cues in their microenvironment, including adhesive molecules that are thought to guide their development. Integrins are the most prominent family of cell adhesive receptors for extracellular matrix (ECM) molecules. Each integrin forms a heterodimer that contains an \( \alpha \) and a \( \beta \) subunit (Hynes, 1992). In vertebrates 18 \( \alpha \) and 8 \( \beta \) subunits have thus far been identified which can form 24 functional integrin receptors (van der Flier and Sonnenberg, 2001). The combination of the \( \alpha \) and \( \beta \) subunits determines ligand-binding specificity, affinity, and intracellular signaling activity of the integrin receptors (Hynes, 1992). \( \beta_1 \) is a prominent subunit, which can associate with 12 \( \alpha \) subunits to form heterodimers. The \( \beta_1 \) subunit can combine with \( \alpha_4 \), \( \alpha_5 \), \( \alpha_8 \), or \( \alpha \), subunits to form heterodimer receptors, which bind to RGD (Arg-Gly-Asp) containing ECM molecules, such as fibronectin and vitronectin (van der Flier and Sonnenberg, 2001).

Integrin ligand binding leads to the formation of focal adhesions where integrins link the ECM to intracellular cytoskeletal complexes and bundles of actin filaments (Critchley, 2000). These protein assemblies play important roles in stabilizing cell adhesion and regulating cell shape and motility. Integrins also mediate transmembrane signal transduction via signaling molecules recruited to focal adhesions (Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999). Protein tyrosine phosphorylation is one of the intracellular events that transmit extracellular cues into
cellular responses (Bauer et al., 1993; Maness and Cox, 1992). For example, tyrosine phosphorylation of focal adhesion kinase and paxillin has been observed in many cell types in response to attachment onto ECM substrates (Burridge et al., 1992). Paxillin is a 68-kDa focal adhesion associated adapter protein implicated in the regulation of integrin signaling and organization of the actin cytoskeleton (Cary and Guan, 1999; Turner, 2000).

Recently, disintegrins have been used as powerful tools to investigate the functional roles of integrins in cell adhesion and signaling (Chavis and Westbrook, 2001; Della Morte et al., 2000; Staiano et al., 1997). The disintegrins are a family of low molecular weight, disulfide-rich, RGD-containing proteins derived from snake venom (Gould et al., 1990). They can bind to integrin receptors on the cell membrane and are potent inhibitors of platelet aggregation and integrin mediated cell adhesion (Dennis et al., 1990). Echistatin is a 5400-Da monomeric disintegrin derived from the venom of the saw-scaled viper, Echis carinatus (Gan et al., 1998). Echistatin expresses an RGD sequence at the apex of the integrin binding loop with four disulfide bonds and is a potent inhibitor of RGD-dependent integrins, including α5β1, αβ3, and αιβ3 (Marcinkiewicz et al., 1996; Smith et al., 2002; Thibault, 2000).

This study is the first to use echistatin to investigate the functional role of integrins during retinal morphogenesis. We have observed that echistatin blocked retinal-derived XR1 glial cell attachment, focal adhesion formation, and integrin-mediated signaling on fibronectin substrates in vitro and also disrupted early retinal development in vivo. These results indicate that integrin-mediated adhesion and signaling are essential for retinal development.

Materials and methods

Animals

Xenopus laevis frogs were obtained from a colony maintained at Iowa State University. Embryos were produced from human chorionic gonadotropin (Sigma-Aldrich, St. Louis, MO)-induced matings and were maintained in 10% Holtfreter’s solution (37 mM NaCl, 0.5 mM MgSO4, 1 mM NaHCO3, 0.4 mM CaCl2, and 0.4 mM KCl) at room temperature. Embryos and larvae were staged according to the normal Xenopus table of Nieuwkoop and Faber (1956). All animal procedures were carried out in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and had the approval of the Iowa State University Committee on Animal Care.

XR1 cell cultures

The XR1 cell line is an immortal glial cell line derived from Xenopus retinal neuroepithelium (Sakaguchi et al., 1989). XR1 cells were grown in tissue culture flasks (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) in 60% L15 media (Sigma) containing 10% fetal bovine serum (Upstate Biotechnology Inc, Lake Placid, NY), 1% embryo extract (Sakaguchi et al., 1989), 2.5 μg/ml fungibact, and 2.5 μg/ml penicillin/streptomycin (Sigma). XR1 cells were detached from subconfluent cultures by exposure to Hank’s dissociation solution (5.37 mM KCl, 0.44 mM KH2PO4, 10.4 mM Na2HPO4, 137.9 mM NaCl, 9.0 mM d-glucose, 0.04 mM Phenol Red) supplemented with 2.5 μg/ml fungibact, 2.5 μg/ml penicillin–streptomycin, 0.2 mg/ml ethylenediamine tetra-acetic acid (EDTA), and 0.5 μg/ml trypsin. Detached cells were collected, pelleted by centrifugation, resuspended in culture media, and seeded onto 12-mm detergent (RBS-35; Pierce, Rockford, IL) washed glass coverslips (Fisher Scientific Co., Pittsburgh, PA) coated with 10 μg/ml fibronectin substrate (Upstate Biotechnology). Cultures were grown at room temperature (~24°C).

Cell adhesion assay

Resuspended XR1 cells were diluted to 1.0 × 10^5 cells/ml after counting and cell viability evaluation with trypan blue exclusion. The cell suspension was plated into 35-mm plastic dishes containing four 12-mm glass coverslips coated with 10 μg/ml fibronectin. Echistatin (Sigma), GRGDSP, or GRGESP peptides (Life Technologies-Gibco BRL, Grand Island, NY) were added into the dishes to final concentrations of 2.5, 5, and 10 μg/ml for echistatin and 50, 100, and 200 μg/ml for the peptides. Control cells received the carrier solution (PBS). The cells were allowed to attach for 30 min and subsequently the cultures were fixed and stained with rhodamine–phalloidin to visualize the attached cells. Images of 16 fields on each of the four coverslips were captured using a 20× objective and the number of adherent cells was counted. The average data from three separate platings were normalized as the percentage of nonadherent cells in treated groups versus the attached cells in the control group to calculate the percentage of inhibition.

Focal adhesion assay

XR1 glial cells were allowed to adhere to fibronectin-coated coverslips for 1 h, and exposed to 2.5 μg/ml echistatin or 100 μg/ml GRGDSP or GRGESP peptides or PBS for 2 h, and fixed and processed for immunocytochemistry with anti-β1 integrin antibody. Cultures were examined using a 40× oil immersion objective. In previous studies, we identified focal adhesions on XR1 cells as discrete streak-like patterns of immunoreactivity where β1 integrins were colocalized with vinculin or phosphotyrosine immunoreactivity at the termini of F-actin filaments (Folsom and Sakaguchi, 1997; Li and Sakaguchi, 2002). As such, in this communication, we have defined focal adhesions as discrete streaks of β1 integrin-IR. Seventy-two objective and the number of adherent cells was counted. The average data from three separate platings were normalized as the percentage of nonadherent cells in treated groups versus the attached cells in the control group to calculate the percentage of inhibition.
sessions. In each field, cells were scored as positive if focal adhesions were present and negative if absent. The proportion of cells displaying focal adhesions was calculated for each group. Data were represented as means ± SEM and analyzed using the Student’s t test.

Cell area measurements

XR1 cultures were examined using a 20× objective and images were captured as described above. Cell area measurements were obtained from captured images using NIH Image 1.58 VDM software (Wayne Rasband, National Institutes of Health, Bethesda, MD). A known distance (100 μm) was measured for calibration, and outlining the cell perimeter produced a calculation of cell area. More than 50 cells from 24 fields of 360 × 280 μm were examined for each condition. Data were represented as means ± SEM and were analyzed using the Student’s t test.

Immunoprecipitation and Western blot analysis

XR1 glial cells were plated onto fibronectin-coated dishes for 1 h, and exposed to 2.5 μg/ml echistatin for 2 h. Cells were scraped from the bottom of the dishes and placed in lysis buffer (0.1 M NaCl, 10 mM Tris, pH 7.6, 1 mM EDTA, 0.2% NP-40, 1 μg/ml aprotinin, 2 mM Na3VO4, and 1 mM PMSF). Samples were homogenized, and protein concentration determined using a Bio-Rad protein assay kit. Protein samples were also obtained from cells in suspension, and also from cells attached for 1 or 3 h on fibronectin-coated dishes. Anti-paxillin antibody was added to the cell lysate, and the preparation was gently rocked at 4°C overnight. A Protein G agarose bead slurry was added and incubated at 4°C for 2 h. Beads were collected by pulsing 5 s in a microcentrifuge at 14,000 rpm, and rinsed three times with ice-cold cell lysis buffer. The agarose beads were resuspended in SDS-sample buffer (0.5 M Tris–HCl, 10% SDS, 10% glycerol, 2.5% bromophenol blue, 5% β-mercaptoethanol). Protein samples were boiled and separated on 7.5% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose, blocked overnight with 1.5% BSA in Tris-buffered saline (TBS, 10 mM Tris–HCl, 150 mM NaCl, pH 8.0), and incubated with antibodies directed against phosphotyrosine for 1 h. Control blots using anti-paxillin antibody were run to confirm equal loading of paxillin in the precipitates. After washing in TBS with 0.1% Tween-20, the membranes were incubated with 1:5000 goat anti-mouse IgG alkaline phosphatase for 45 min. The blots were visualized with NBT/BCIP staining procedures (Promega, Madison, WI). Densitometric analysis was performed with NIH Image 1.58 VDM software.

In vivo treatment with echistatin

Embryos between stages 23–25 were anesthetized by immersion in 100% modified Ringer’s solution containing 1:10,000 MS222. Embryos were immobilized on their right side in a sylgard-coated dish by using stainless-steel minut pins. The skin overlying the left optic vesicle was carefully removed, and the embryos placed in Holtfreter’s solution with 2.5 μg/ml fungibact and 2.5 μg/ml penicillin-streptomycin in the presence of 10 μg/ml echistatin or carrier solution for 48 h up to St 40. Five animals at St 40 from each group (treated and control) were transferred into Holtfreter’s solution and allowed to develop up to St 47 in the absence of the echistatin. The eyes from all the tadpoles were processed for immunohistochemical analysis.

Antibody injection procedure

Embryos between stages 23 and 25 were anesthetized by immersion in 100% modified Ringer’s solution containing 1:10,000 MS222. Embryos were immobilized on their right side in a sylgard-coated dish by using stainless-steel minut pins. The skin overlying the left optic vesicle was carefully removed. A glass micropipette containing injection solutions (1 mg/ml β1 integrin function blocking antibody, 3818 or control nonspecific preimmune antibody) was carefully maneuvered into position, and injection into the optic vesicles were made using a Picospritzer microinjection apparatus (General Valve Corp.). Microelectrodes were made from capillary electrode glass (Fredrich Hare Co.) using a vertical pipette puller (David Kopf Instruments). The volume of injected antibody was approximately 10–20 nl. Following injection, the embryos were placed in Holtfreter’s solution with 2.5 μg/ml fungibact and 2.5 μg/ml penicillin-streptomycin and allowed to survive for 48 h.

Immunohistochemistry

Xenopus larvae and cultured cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h (animals) or 30 min (cells). The specimens were rinsed with buffer and cryoprotected in 30% sucrose in 0.1 M P04 buffer overnight, and then frozen in OCT medium (Tissue-Tek, Sakura Finetek U.S.A., Inc. Torrance, CA). The frozen tissues were sectioned at 16 μm using a cryostat (Reichert HistoSTAT) and sections were thaw mounted on Superfrost microscope slides (Fisher). For antibody labeling procedures, the tissue sections and cultures were rinsed in phosphate-buffered saline (PBS, 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na2HPO4, 1.47 mM KH2PO4) and blocked in 5% goat serum, containing 0.4% BSA and 0.2% Triton X-100 in PBS. Primary antibodies were diluted in blocking solution and preparations incubated overnight at 4°C. On the following day, the preparations were rinsed with PBS and incubated with appropriate
secondary antibodies conjugated to Alexa 488 or RITC (diluted 1:200 in blocking solution) for 90 min at room temperature and subsequently rinsed and mounted under glass coverslips. For double-labeling immunocytochemistry, an Alexa-488-conjugated goat anti-mouse IgG or a biotinylated goat anti-mouse IgG (1:300, Vector Laboratories Inc.) and avidin-AMCA (1:1000, Vector laboratories Inc.) were used following the second primary antibody incubation. These preparations were subsequently triple-labeled with rhodamine–phalloidin (1:300, 30 min from Molecular Probes, Eugene, OR) to visualize the F-actin cytoskeleton. As a control, single-label studies were performed parallel to the multilabeling studies to rule out that similar patterns were due to bleed-through and the other fluorescence channels were also examined to ensure that no bleed-through occurred. Negative controls were performed in parallel by omission of the primary or secondary antibodies. No antibody labeling was observed in the controls.

**Antibodies**

β1 integrin receptors were identified using monoclonal antibody 8C8, purchased from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) and diluted 1:10 with blocking solution and polyclonal anti-β1 integrin (3818, a gift from Dr. K. Yamada, Lab of Molecular Biology, NCI, Bethesda, MD, diluted to 20 µg/ml). Anti-paxillin, clone 439 (Transduction Laboratory), was diluted at 1:100. Anti-phosphotyrosine monoclonal antibody, 4G10 (Upstate Biotechnology Inc.), was diluted at 1:200. Photoreceptors were identified using anti-Xenopus photoreceptor antibody, XAP-1, diluted at 1:20 (Sakaguchi et al., 1997). Anti-synaptic vesicle protein SV2 antibody (Developmental Studies Hybridoma Bank, University of Iowa) was diluted at 1:20, and Xenopus anti-neuronal antibody, XAN-5, was diluted at 1:50. Goat anti-mouse IgG, IgM, or goat anti-rabbit IgG secondary antibodies conjugated with RITC or Alexa 488 were purchased from Southern Biotechnology (Birmingham, AL) or Molecular Probes, respectively.

**Analysis of fluorescence images**

Tissue sections and cultured cells were examined using a Nikon Microphot-FXA photomicroscope (Nikon Inc. Garden City, NY) equipped with epifluorescence. Images were captured with a Kodak Megaplus 1.4 CCD camera connected to a Percepts Megagrabber framegrabber using NIH Image 1.58 VDM software in a Macintosh computer (Apple Computer, Cupertino, CA). Analysis of multilabeled tissues was performed on a Leica TCS-NT confocal scanning laser microscope (Leica Microsystems, Inc., Exton, PA). Figures were prepared on a Macintosh computer (Apple Computer) using Adobe Photoshop version 7.0 and Macromedia Freehand version 10.0 for Macintosh.

**Results**

*Echistatin inhibits XR1 retinal glial cell attachment to fibronectin*

XR1 glial cells serve as an ideal cell system to investigate β1-integrin-mediated cell adhesion with respect to the developing *Xenopus* visual system (Folsom and Sakaguchi, 1997; Li and Sakaguchi, 2002; Li et al., 2004). The XR1 glial cells were derived from the *Xenopus* retinal neuroepithelium (Sakaguchi et al., 1989) and have been shown to express at least αv, α5, and β1 integrin subunits (Folsom and Sakaguchi, 1997; Sakaguchi, unpublished observation). Integrin αv/β1 is a receptor for fibronectin and vitronectin, while αv/β3 is a receptor for fibronectin. Both fibronectin and vitronectin contain the classic integrin binding motif, the RGD sequence.

To investigate the importance of β1 integrins during retinal development, we have employed echistatin, a disintegrin that is a potent inhibitor of RGD dependent integrins, including αvβ1 and αvβ3 (Staiano et al., 1995; Yang et al., 1996). To identify whether echistatin and RGD-containing peptides could disrupt integrin-fibronectin interaction in XR1 cells, a cell adhesion assay was performed. XR1 glial cells were plated onto fibronectin-coated coverslips in the presence of different concentrations of echistatin or GRGDSP peptides. Cells treated with GRGESP peptides or PBS served as controls. Fig. 1 illustrates the inhibitory
Echistatin disrupts retinal lamination: an in vivo perturbation analysis

β₁ integrins and focal adhesion associated proteins have been identified to be differentially regulated during Xenopus retinal development (Li and Sakaguchi, 2002; Li et al., 2004). To investigate the role of β₁-integrin-mediated adhesion and signaling during early retinal development in vivo, we bath-applied echistatin to the optic vesicle. The exposed eye preparation, similar to the exposed brain preparation (Chien et al., 1993; McFarlane et al., 1995; Worley and Holt, 1996), permitted direct access of the echistatin to the optic vesicle. At the optic vesicle stage, the
retina is a relatively undifferentiated neuroepithelium. These embryos were incubated in the presence of echistatin until stage 40, when the retina is normally well differentiated, exhibiting its distinct laminar organization.

Embryos incubated with echistatin appeared healthy and developed at a normal rate when compared to control embryos. However, those eyes exposed to echistatin displayed severe defects in the pattern of retinal lamination.

Fig. 2. Echistatin disrupts focal adhesion assembly in retinal-derived XR1 glial cells. Fluorescence photomicrographs reveal the disruption of focal adhesions and the F-actin stress fibers following echistatin treatment of XR1 glial cells. XR1 cells were plated for 1 h and then incubated with 2.5 μg/ml echistatin or 100 μg/ml peptides for 2 h. Focal adhesions were identified with β1 integrin antibody (A, E, I, M), and paxillin antibody (B, J) or phosphotyrosine antibody (F, N). F-actin filaments were labeled with rhodamine phalloidin (C, G, K, O). The merged images (D, H, L and P) illustrate colocalization of the β1 integrin receptors with paxillin (D) or phosphotyrosine (H) at the termini of the actin filaments. Note that β1 integrin-, paxillin-, and phosphotyrosine-IRs were absent from focal adhesions and actin stress fibers were not well organized in echistatin-treated cells (I–P) compared with the control (A–H). Abbreviations: P-Tyr, phosphotyrosine; Ech, echistatin. Scale bar = 20 μM.
Retinal histogenesis was analyzed with anti-synaptic vesicle protein SV2 antibody (SV2) and anti-photoreceptor protein antibody (XAP-1). Synaptic vesicle protein SV2 is a transmembrane transporter in vesicles that are located predominantly to the nerve terminal (Feany et al., 1992), while XAP-1 protein correlates with inner and outer segment assembly of photoreceptors (Wohabrebbi et al., 2002). In control retinas, the XAP-1 antibody labeled the discrete band of photoreceptor outer segments (Fig. 5A), while SV2 antibody clearly demarcated the outer plexiform and inner plexiform layers (OPL and IPL, respectively) (Fig. 5B). In 20 out of 23 echistatin-treated retinas, retinal lamination was disrupted, particularly in the outer retina (Table 1; Figs. 5D–L). In all the defective retinas, ectopic photoreceptors were observed usually forming circular clusters of cells similar to rosette structures (Figs. 5D,G), and XAP-1-IR was no longer continuous in the row of outer segments of the photoreceptors (Fig. 5D). The lumen of the rosette structures appears tubular and was formed by photoreceptor outer segments as determined by XAP-1-IR. Moreover, ectopic plexiform layers were observed surrounding the rosette structures (Figs. 5E,F,H,I,K,L). Five echistatin-treated animals were subsequently allowed to develop to St 47 in normal rearing solution in the absence of the echistatin. Some of the effects of the blockade appear to be mitigated as development proceeds to St 47. However, four of these animals displayed rosette structures. In general, the rosettes were fewer per retina and are smaller. In addition, fewer defects in the normal photoreceptor layer were observed in these animals that were allowed to survive to St 47 (Figs. 5J–L). The defects in these retinas appeared less severe compared with the defects in the treated retinas examined at St 40.

Fig. 3. Echistatin blocks focal adhesion assembly and XR1 cell spreading. XR1 cells were allowed to attach and spread for 1 h and subsequently incubated with 2.5 μg/ml echistatin or 100 μg/ml peptides in the culture media for 2 h. (A) Echistatin inhibited focal adhesion assembly. Focal adhesions were identified with β1 integrin-IR. The values were expressed as the percentage of cells displaying focal adhesions from three experiments. At least 150 cells were counted for each treatment. (B) Echistatin inhibited glial cell spreading. Cell area measurements were obtained from captured images (n = 50) with NIH image 1.58 VDM software. Error bars represent ± SEM; *, Statistically significant at P < 0.01.

Fig. 4. Echistatin reduces paxillin phosphorylation in fibronectin-adherent XR1 glial cells. XR1 cells were in suspension (S) for 1 h or allowed to adhere to fibronectin coated dishes for 1 h and then exposed to 2.5 μg/ml echistatin for 2 h. Cell lysates were immunoprecipitated with anti-paxillin antibody and subsequently separated by electrophoresis. After blotting, tyrosine phosphorylated proteins were probed with anti-phosphotyrosine antibody (A), and paxillin was probed with anti-paxillin antibody (B). (C) The relative levels of phospho-paxillin. The absorbance of bands corresponding to phosphorylated paxillin in (A) was determined by densitometric analysis and the values on the y-axis represent the means ± SEM in arbitrary units from three separate experiments of identical design. The absorbance values of paxillin bands in B were with less than 7% difference.
An $\beta_1$ integrin function blocking antibody has been identified to inhibit neurite outgrowth of embryonic *Xenopus* retina and XR1 glial cell spreading, as well as to disrupt the formation of the *Xenopus* retinotectal projection (Sakaguchi and Radke, 1996; Stone and Sakaguchi, 1996). To further investigate the role of $\beta_1$-integrin-mediated adhesion and signaling during early retinal development in vivo, we microinjected the $\beta_1$ integrin function blocking antibody into the optic vesicle. Embryos injected with the integrin and control antibodies appeared healthy and developed at a normal rate when compared to control embryos. However, those eyes injected with the $\beta_1$ integrin antibody displayed severe defects in the pattern of retinal lamination. Retinal histogenesis was analyzed with *Xenopus* anti-neuronal antibody, XAN-5 and anti-photoreceptor protein antibody, XAP-
Table 1

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a Stage 25 embryos were treated with echistatin or microinjected with anti-β1 antibody and allowed to survive for 48 h to stage 40.

b Twenty-one control animals were treated with carrier solution.

c Twenty-eight animals were treated with echistatin and five of these echistatin-treated animals were subsequently allowed to develop to St 47 in the absence of echistatin.

d Ten animals were injected with β1 integrin function blocking antibody, while five were injected with preimmune antibody. Immunohistochemical analysis was performed on retinal sections with anti-synaptic vesicle protein (SV2) or anti-neuronal antibody (XAN-5), and anti-photoreceptor protein (XAP-1) antibodies.

1. In the retinas injected with control antibody, the labeling pattern for XAN-5 antibodies clearly demarcated the OPL and IPL (Fig. 6B), while the XAP-1 antibody labeled the discrete band of photoreceptor outer segments (Fig. 6C). In the retinas injected with β1 integrin function blocking antibody, retinal lamination was disrupted; rosette structures with ectopic photoreceptors and plexiform layers were formed in outer retina and XAP-1-IR was no longer restricted to a continuous band of photoreceptor outer segments (Table 1; Figs. 6E–H). These defective phenotypes are similar to the malformations observed following echistatin treatment.

Discussion

Cell adhesion occurs in three stages: attachment, spreading, and formation of focal adhesions and actin stress fibers (Burrage et al., 1988). Focal adhesions, characteristic of strong cell adhesion, consist of clustered integrins and associated structural and signaling molecules that link the ECM and actin cytoskeleton (Jockusch et al., 1995). At the initial stage, cell attachment involves the interactions between integrins and ECM substrates, and the integrin activation induces integrin clustering and increases integrin affinity. At the intermediate stage, cells increase their surface contact area on the ECM substrates through cell spreading. These events lead to the formation of focal adhesions and stress fibers, which requires appropriate extrinsic and internal signals (Hughes and Pfaff, 1998; Humphries, 1996; Schoenwaelder and Burrage, 1999). Through focal adhesions and stress fibers, integrins bidirectionally transmit mechanical and biochemical signals that are extracellular and intracellular in origin (Giancotti and Ruoslahti, 1999; Howe et al., 1998).

Both echistatin and GRGDSP peptides inhibited XR1 cell attachment to fibronectin, but echistatin was about 700-fold more effective than RGD-containing peptides at inhibiting cell attachment. This is consistent with other studies in which echistatin was shown to be about 200 times more potent than RGD-containing peptides in inhibiting RPE cell attachment to fibronectin substrates, and about 1000 times more effective at inhibiting platelet aggregation (Gould et al., 1990; Yang et al., 1996). Furthermore, echistatin effectively blocked cell spreading and focal adhesion formation in Xenopus XR1 glial cells. In the presence of 2.5 μg/ml echistatin, XR1 cells began retracting their fringing edges, and began rounding up and cell detachment was occasionally observed, while in the presence of a higher concentration of echistatin, a large proportion of the cells detached from the coverslips. However, GRGDSP peptides at 100 μg/ml were relatively ineffective in blocking cell spreading and focal adhesion formation. This difference of inhibitory effect is most likely due to the different configuration of the molecules. The optimal conformation of the RGD loop, as well as the amino acid sequences flanking the RGD locus in echistatin, determine the specificity and affinity against the RGD dependent integrins (Marcinkiewicz et al., 1997; McLane et al., 1996; Smith et al., 2002; Wierzbicka-Patynowski et al., 1999).

Echistatin has been shown to bind with high affinity to α5β1 and α3β1 integrin receptors (Kumar et al., 1997; Wierzbicka-Patynowski et al., 1999) as well as α5β1, α5β1, and α3β1 integrins (Thibault, 2000). All these integrin receptors can interact with fibronectin. In addition to the β1 subunit, α1 and α5 subunits are expressed in XR1 glial cells.
It is likely that echistatin competes for integrin receptors at the cell surface, and focal adhesions organized by \( \beta_1 \) containing integrins such as \( \alpha_5\beta_1 \) and \( \alpha_v\beta_1 \) may represent the privileged site of its action. Other integrins like \( \alpha_3\beta_1 \), \( \alpha_8\beta_1 \) and \( \alpha_v\beta_3 \) may be involved in the interactions; thus, in future studies, it will be important to fully identify the complement of \( \alpha \) and \( \beta \) subunits present in the XR1 cells. Staiano et al. (1997) have reported that echistatin caused disassembly of focal adhesions and detachment of well attached melanoma cells under serum-free medium. Under serum-free condition, GRGDS peptides could also disrupt focal adhesion formation in XR1 cells (data not shown). It is likely that RGD-containing peptides at moderate concentration can inhibit the initial stage of adhesion or weak adhesion without facilitation of other attenuating signals. After the cells were plated for more than 3 h, the cells were well attached and the application of echistatin had a decreased inhibitory effect on the focal adhesions compared with application after the cells were plated for 1 h. The inhibition of focal adhesions by echistatin is specific and dose-dependent. Echistatin at the range of concentrations used was not cytotoxic to XR1 cells, and the inhibition was reversible. Furthermore, echistatin at a lower dose of 0.5 \( \mu \)g/ml had much less effect on the focal adhesions. Echistatin at 0.5 \( \mu \)g/ml blocked about 50% of cell attachment and 10% of XR1 cell focal adhesions (data not shown).

**Echistatin blocks integrin-mediated signaling**

In addition to the inhibition of focal adhesion formation, echistatin reduced the tyrosine phosphorylation levels of paxillin. Paxillin, a focal adhesion associated adapter protein, is implicated in the regulation of integrin signaling (Turner, 2000). The decrease of paxillin phosphorylation indicates that echistatin inhibited integrin signaling in XR1 cells. Ligand binding promotes the conformational change that allows intracellular interactions of integrin tails with cytoskeletal molecules and induces the formation of focal adhesions and initiates cell signaling (Cary and Guan, 1999; Clark and Brugge, 1995). For example, in many types of cells, attachment to ECM substrates causes an increase of...
phosphorylation of focal adhesion kinase pp125FAK and paxillin (Burridge et al., 1992; Cattelino et al., 1997). Adhesion of XR1 cells to fibronectin substrates induces a rapid increase of tyrosine phosphorylation of paxillin. Echistatin interactions with engaged integrins on the cell surface could lead to a conformational change that inactivates integrin molecules or reverses the adhesion process. This disruption of integrin-mediated adhesion may result in a subsequent blocking of signaling cascades, including tyrosine dephosphorylation and disassembly of focal adhesions and actin stress fibers. The Staiano group has reported that exposure of melanoma cells to echistatin inhibits paxillin and FAK phosphorylation and causes a dramatic disassembly of focal adhesions with disappearance of both FAK and paxillin (Della Morte et al., 2000; Staiano et al., 1997).

Echistatin and β1 integrin function blocking antibody disrupts retinal development

Perturbation studies have shown that integrin-mediated selective adhesion plays a critical role in regulating cellular processes during early development (Darribere et al., 2000). Recently, echistatin, GRGDSP peptides, and integrin function blocking antibody were reported to block synaptic maturation at hippocampal synapses in vitro (Chavis and Westbrook, 2001). Our study demonstrates that application of echistatin and β1 integrin function blocking antibody to early embryonic retina disrupted retinal lamination and induced rosette structures with ectopic photoreceptors in outer retina. Ectopic plexiform layers between the original outer plexiform layer and the rosette structures were also observed. The mechanisms by which rosettes are produced are not clear. The rosettes are structural anomalies and it is likely they are products of abnormal cell proliferation, differentiation, or migration, or a combination of these processes. It is possible they are produced by a localized overgrowth of the nascent outer nuclear layer or by atypical differentiation and migration of progeny from retinal stem cells. The rosettes appear to be formed mainly of ONL cells arranged radially around a lumen. In echistatin-treated retinas, XAP-1 immunoreactivity was absent from some areas of the outer retina, where photoreceptors should be located. In addition, infoldings of XAP-1-IR were often observed in the treated retinas. However, when the treated animals were allowed to recover and develop to St 47 in the absence of the echistatin, only one or two rosette structures were observed in each treated retina, the size was much smaller relative to the retina and no abnormal photoreceptor infolding or discontinuous XAP-1-IR was observed in the outer retina. Thus, it seems likely that the disruption of the interactions between integrins and the ECM lead to invagination of retinal progenitors and creation of the anomalous rosette structures.

Rosettes are characteristic structures that are of great concern in developmental biology and medicine, because they have also been observed in retinoblastomas, naturally occurring malformations or in grafts of transplanted embryonic retinas (Bogemann, 1986; Liu et al., 1983; Ohira et al., 1994; Seiler et al., 1995). In dissociated chick retinal cultures, rosette structures were formed; however, Müller glial cells or Müller cell-derived factors, as well as RPE cells, could reorganize dissociated cells into appropriately laminated retinal structures (Rothermel et al., 1997; Willbold et al., 2000). This indicates that the cell–cell and cell–ECM interactions may have an important role in organizing and maintaining the columnar organization of the retina. Furthermore, β1 integrin antibodies and RGD peptides have been shown to disrupt eye morphogenesis after being micro-injected into preoptic regions of chick embryos (Svennevik and Linser, 1993). Injection of β1 integrin function blocking antibodies into Xenopus optic vesicles also appears to lead to formation of similar rosette structures in the retinas. Moreover, in approximately one third of the rosettes, we observed a patch of pigmented cells that had invaded these anomalous structures. This is a hallmark associated with retinitis pigmentosa. As such, these results suggest a role for ECM–integrin interactions in naturally occurring retinal pathologies. Thus, the induction of retinal rosettes by echistatin and function blocking integrin antibodies is likely due to the disruption of integrin-mediated cellular interactions between retinal stem/progenitor cells and the surrounding ECM.

A growing body of evidence suggests that cell–cell and cell–ECM interactions are essential in many phases of neural development, including neuroblast migration, determination of cell fate, axon outgrowth, and synapse formation (Clegg et al., 2000). For example, different laminin isoforms are expressed in multiple locations in the retina, and laminin-3 (s-laminin) appears to be involved in photoreceptor determination, inner and outer segment development, and photoreceptor synaptogenesis (Hunter et al., 1992; Libby et al., 1996). Laminins also contain RGD sequences, but are normally cryptic and inaccessible unlike fibronectin and vitronectin. Thus far, β1, α5, and α5 integrin subunits have been identified in Xenopus retina (Li and Sakaguchi, 2002; Sakaguchi unpublished observations). α5 to α6 subunits are expressed in the tiger salamander retina (Sherry and Proske, 2001) and αα, αβ, and β subunits have been identified in the chick retina (Cann et al., 1996; Gervin et al., 1996). It is likely that all of these subunits are also expressed in Xenopus retina. Echistatin may also disrupt other RGD-dependent integrin receptors containing β3 and β5 subunits. In addition to photoreceptor development, echistatin possibly disrupts other retinal cells, altering their positioning and synaptogenesis. Single cell recording and further cellular analysis would shed additional light on the importance of ECM–integrin signaling during retinal development.

Echistatin and β1 integrin function blocking antibody disrupt retinal lamination. These results provide compelling evidence that integrin-mediated adhesion and signaling play a decisive role in determining the position and polarity of retinal cells as well as regulating retinal morphogenesis during development.
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