Inhibition of Retinal Pigment Epithelial Cell-Induced Tractional Retinal Detachment by Disintegrins, A Group of Arg-Gly-Asp-Containing Peptides From Viper Venom

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Purpose. Integrin-mediated extracellular matrix (ECM) attachment plays an important role in vitreous contraction of retinal pigment epithelial (RPE) cells. Disintegrins, a group of Arg-Gly-Asp (RGD)-containing peptides from viper venom, are potential anti-adhesion agents that interfere with integrin–ECM binding. This study was performed to determine whether disintegrins were effective in inhibiting RPE cell-induced matrix attachment in vitro and tractional retinal detachment in a rabbit model in vivo.

Methods. Two disintegrins, echistatin from viper Echis carinatus and flavoridin from Trimeresurus flavoviridis, were used. The expression of integrins on the surface of bovine and rabbit RPE cells was examined by indirect immunofluorescent stain with specific anti-integrin monoclonal antibodies. The inhibitory effect of disintegrins on RPE cell-mediated ECM attachment and vitreous contraction was evaluated with cell adhesion and vitreous contraction assays. In the in vivo model, rabbit eyes were injected intravitreally with either homologous rabbit RPE cells alone or together with disintegrins to induce tractional retinal detachment. The cytotoxic effect of disintegrins was examined with a cell proliferation assay using the alamar blue method. Retinal toxicity of disintegrins was evaluated with electroretinograms and histologic examination of the rabbit eyes.

Results. Bovine and rabbit RPE cells showed the positive staining for the integrins α2β1 and αcβ1 on cell surface. Disintegrins, echistatin, and flavoridin inhibited RPE cell attachment to the ECM. The potency of disintegrins was 150 to 300 times higher than that of Gly-Arg-Gly-Asp-Ser (GRGDS) peptide. The disintegrins also inhibited RPE cell-induced vitreous contraction in a dose-dependent manner, whereas the GRGDS peptide had no effect. In the in vivo experiment, echistatin (50 μg/ml) or flavorin (80 μg/ml) significantly inhibited RPE cell-induced tractional retinal detachment compared with the control group at week 2 (P < 0.05) and week 4 (P < 0.01) after surgery. Disintegrins were nontoxic to RPE cells and rabbit retina as evaluated by cytotoxicity tests, electroretinograms, and histologic examinations.

Conclusions. The disintegrins were effective in inhibiting RPE cell attachment to the ECM and vitreous contraction in vitro. They also were effective in suppressing RPE cell-induced tractional retinal detachment in the rabbit eyes. They were nontoxic. Disintegrins and their analogs might be potential anti-adhesion therapeutic agents in the treatment of proliferative vitreoretinopathy.

Implications. Disintegrins and their analogs might be potential anti-adhesion therapeutic agents in the treatment of proliferative vitreoretinopathy, which is caused by the formation of proliferating fibrocellular membrane on both sides of retinal surfaces and in the vitreous cavity. Further contraction of the fibrocellular membrane causes tractional retinal detachment (TRD). Retinal pigment epithelial (RPE) cells, fibroblasts, glial cells, macrophages, and myofibroblast-like cells are found in the proliferating membrane. They are surrounded by various amounts of extracellular matrix (ECM), including collagen, fibronectin, vitronectin, and laminin.
With the advancement of vitreoretinal surgery, the prognosis of PVR has been improved. Long-term success, however, often is prevented by the recurrence of proliferative contractile membranes. Repeated surgery often exacerbates the inflammatory reaction within the vitreous cavity. Pharmacologically, various drugs, including antimetabolites, colchicine, and steroids, have been tested as potential therapeutic agents for the treatment of PVR. However, many of the inhibitors of cellular proliferation have side effects that are toxic to the eye. Thus, further understanding the pathogenesis of PVR and searching for more appropriate and effective therapeutic agents are critical.

Much progress has been made in understanding the pathogenesis of PVR in the recent decades. Basically, PVR is considered an aberrant wound healing process in the ocular tissue. A final common pathway is suggested to be involved in cell-mediated TRD. A large body of evidence points out that RPE cells play a central role in the formation of PVR. When released from their normal anatomic position, RPE cells contact the vitreous and change their morphology and cellular function. They are stimulated subsequently by inflammatory mediators, such as, cytokines, that are derived from serum or are synthesized locally by lymphocytes, platelets, and RPE cells themselves. At the same time, ECM components, including collagen, fibronectin, vitronectin, and laminin, are synthesized and deposited on the retinal surfaces. Of these stages, attachment of RPE cells to ECMs probably is the most important. Activation, migration, and proliferation can occur only after RPE cells establish attachment. Cellular surface adhesion molecules mediating cell–cell and cell–ECM interaction participate in these processes.

Integrins are cell surface receptors for ECM proteins that mediate cell adhesion and migration. These receptors consist of two noncovalently linked α and β subunits and are associated with contractile elements of the cytoskeleton at focal adhesions on the plasma membrane. The interaction between integrins and the ECM involves both Arg-Gly-Asp (RGD) cell-binding sequences and non-RGD cell-binding sequences within ECM macromolecules. These receptors play an important role in the processes of embryogenesis, wound healing, thrombosis, immune reactions, and tumor metastasis. Several studies have been conducted to identify the integrins expressed on the RPE cells and PVR membranes. Because cell–ECM interaction is important in the processes of wound healing, one could predict that integrins would be actively involved in the pathogenesis of PVR.

Recently, a number of low molecular weight, RGD-containing, cysteine-rich peptides have been isolated from the venom of various vipers. These peptides represent a new class of protein called disintegrins, because they interfere with the interaction of adhesive ligands with their integrin receptors. One study showed that the potency of disintegrins is 500 to 2000 times higher than that of short RGDX peptides in inhibiting platelet aggregation. Echistatin, derived from the venom of the saw-scaled viper Echis carinatus, is a single-chain polypeptide of 49 amino acid residues with a molecular weight of 5400 Da. Another disintegrin, flavoridin, derived from the venom of Trimeresurus flavoviridis, is a single polypeptide of 70 amino acid residues with a molecular mass of 7600 Da. Both disintegrins are potent platelet aggregation inhibitors that interfere with the binding of fibrinogen with the platelet membrane glycoprotein IIb–IIIa complex. Several reports have shown that they are effective in preventing coronary arterial thrombosis in canine models, inhibit platelet adhesion in extracorporeal circuits, inhibit bone resorption by osteoclasts in culture, and prevent melanoma cell metastasis to lung in mice.

The purpose of this study was to investigate whether the disintegrins echistatin and flavoridin have any inhibitory effect on integrin-mediated RPE cell attachment to the ECM and vitreous gel contraction in vitro. We also characterized the inhibitory effect of disintegrins to the cell-mediated TRD in a well-established animal model of PVR.

MATERIALS AND METHODS
Reagents
Cell culture reagents, including RPMI-1640, Dulbecco’s modified Eagle’s medium, Hanks’ buffered salt solution, fetal calf serum, trypsin—ethylenediaminetetraacetic acid (EDTA), penicillin—streptomycin, and fungizone were purchased from Gibco (Grand Island, NY). Tissue culture plates and flasks were obtained from Nunc (Roskilde, Denmark). Extracellular matrix, including fibronectin (from bovine plasma), vitronectin (from human plasma), type I collagen (from calf skin), type IV collagen (from mouse sarcoma), and laminin (from basement membrane of mouse sarcoma) were purchased from Sigma Chemical (St. Louis, MO), as were GRGDS peptide, echistatin, and flavoridin. Monoclonal antibodies against integrin subunit β1 (clone DF5) was from Chemicon (Temecula, CA), α2 (clone P1E6) was from Oncogene Science, (Uniondale, NY), and α5 (clone SAM1) was from Immunotech (Westbrook, ME). Anti-cytokeratin monoclonal antibodies (clone AE1/AE3) were from Boehringer (Indianapolis, IN). Fluorescein isothiocyanate-conjugated goat anti-mouse antibody was obtained from Sigma. Alamar blue dye was purchased from Alamar Bioscience (Sacramento, CA).
Bovine Retinal Pigment Epithelial Cell Culture

The method of Basu et al.27 for preparing bovine RPE cell culture was followed. In brief, fresh bovine eyes were obtained from slaughterhouse within 6 hours of death. Under sterile conditions, a circumferential incision was made in the eye around the equator, and then the cornea, lens, vitreous, and retina were removed. Trypsin (0.25%) with EDTA (0.02%) was added to the remaining cup and incubated at 37°C for 25 minutes. Then the RPE cells, loosened from the Bruch’s membrane, were pipetted out. The cell suspension was centrifuged at 200g for 10 minutes and washed twice with culture medium. The culture medium consisted of RPMI-1640 with 25 mM Hepes buffer, 10% fetal calf serum, penicillin 100 U/ml, streptomycin 100 /g/ml, and fungizone 0.25 /g/ml. Cells were resuspended in 10 ml of culture medium in a 25-mm2 plastic flask and incubated at 37°C under 5% CO2. When RPE cells in the culture reached confluence, cells were treated with trypsin–EDTA and subcultured. Fifth- to tenth-passage RPE cells were used in the experiment. Immunohistochemical staining for cytokeratin with the monoclonal antibodies of clones AE1 and AE3 was performed to confirm the presence of RPE cells.

Rabbit RPE cells culture has been described previously.28 Animal experiments were carried out according to guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. New Zealand albino rabbits, each weighing approximately 2.5 kg, were killed by intravenous injection of excessive doses of pentothal. Globes were enucleated immediately. Culture procedures were similar to those for bovine RPE cell culture except that the culture medium consisted of Dulbecco’s modified Eagle’s medium with L-glucose (4500 mg/ml), 25 mM Hepes buffer, and L-glutamine supplemented with 20% fetal calf serum, antibiotics, and antimycotics. The cells were incubated at 37°C under 5% CO2. The medium was changed after 5 days and then twice weekly. When the cells in the culture reached confluence, they were subcultured, and cells of the early passage (five passages) were used in the following in vivo experiment.

Localization of Integrins by Indirect Immunofluorescent Stain

Retinal pigment epithelial cells grown on coverslips were fixed with 0.07% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes. After washing with PBS, cells were incubated with 20% normal goat whole serum with 4% bovine serum albumin in PBS at 37°C for 30 minutes to block nonspecific binding. After extensive rinsing, cells were incubated with monoclonal anti-integrin subunit antibodies at 37°C for 60 minutes. After washing, cells were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse antibody at 37°C for 60 minutes. In negative control, the primary antibody was omitted. The slides were observed and photographed with fluorescence microscopy.

Bovine Retinal Pigment Epithelial Cell Adhesion Assay

Bovine RPE cells were detached with trypsin–EDTA and washed with RPMI to remove residual fetal calf serum. Cells were resuspended in RPMI at a concentration of 1 X 10^6 cells/ml. Ninety-six well tissue culture plates had been coated overnight at 4°C with 50 /l of fibronectin (30 /g/ml), vitronectin (15 /g/ml), laminin (15 /g/ml), collagen type I (80 /g/ml), and collagen type IV (80 /g/ml) in PBS. A 200- /l aliquot of cells, pretreated with various concentrations of peptides for 30 minutes, was placed into each well and incubated for 90 minutes at 37°C. Nonadherent cells were removed by aspiration, and the attached cells were washed gently with PBS. Adherent cells were fixed with 2% glutaraldehyde for 10 minutes and then stained with 2% Giemsa solution for 20 minutes. Cells were viewed at 100X magnification using an inverted phase-contrast microscope. Cells were counted with a 1-mm2 reticle in the eyepiece. The experiments were conducted in quadruplicate and were repeated at least three times.

Vitreous Gel Contraction Assay

The bovine vitreous explant model was adopted from Forrester et al.29 In brief, fresh bovine eyes were obtained from the slaughterhouse and used within 6 hours of death. Under sterile conditions, the anterior segment of the eye was removed and intact vitreous gel was dissected from the retina. The vitreous gels were dialyzed for at least 24 hours against culture medium. Pieces of dialyzed vitreous (0.8 to 1.2 ml) were cut and placed in a 24-well culture plate. One hundred microliters of bovine RPE cell suspension containing 1 X 10^6 cells, pretreated with various concentrations of peptides for 30 minutes, were placed on the convex surface of the vitreous for 15 minutes before the further addition of medium. Cells were incubated at 37°C. To estimate the volume change of the vitreous gel induced by RPE cells before and after cell culture, gels were dehydrated and placed in a known volume of fluid in a volumetric cylinder, and the volume of the gel was calculated from readings of the displacement in the fluid level. For evaluation of the inhibitory effect of peptides on the vitreous gel contraction, various concentrations of peptides were preincubated with RPE cell suspension for 90 minutes and then added to the vitreous gel. Changes in the vitreous volume were measured under sterile conditions 24 hours later. Gels were returned to the culture plate.
to incubate for another 48 hours; then they were used for the volume measurement.

**Experimental Model of Proliferative Vitreoretinopathy**

New Zealand albino rabbits, each weighing between 2 and 2.5 kg, were used. All animals were sedated by intramuscular injections of ketamine–xylazine (10 mg/kg body weight). The eyes were dilated with one drop of 1% cyclopentolate and 2.5% phenylephrine. Using a 30-gauge needle and a tuberculin syringe, an anterior chamber paracentesis of approximately 0.1 ml aqueous humor was drained. This made the globe soft and lowered the intraocular pressure so that loss of cells from reflex after intravitreal injection could be prevented. Homologous rabbit RPE cells were trypsinized, centrifuged, and resuspended in Hanks’ buffered salt solution with a final concentration of 2.5 × 10^5 cell/ml. Viability, as determined by the trypan blue exclusion test, was always higher than 95%. Cells were preincubated with GRGDS peptide, flavoridin, or echistatin for 30 minutes before injection. Twenty-five eyes of 25 rabbits were divided into five groups. Group one (n = 5) was used as a control and was given injections of 250,000 cell/0.1 ml without any peptides. Group two (n = 5) was injected with 250,000 cells and 1000 µg/ml GRGDS in 0.1 ml. Group three (n = 5) was injected with 250,000 cells and 10 µg/ml flavoridin in 0.1 ml. Group four (n = 5) was injected with 250,000 cells and 80 µg/ml flavoridin in 0.1 ml. Group five (n = 5) was used as a control and was given injection of 250,000 cell/0.1 ml. Viability, as determined by the trypan blue exclusion test, was always higher than 95%. Cells were preincubated with GRGDS peptide, flavoridin, or echistatin for 30 minutes before injection. Twenty-five eyes of 25 rabbits were divided into five groups. Group one (n = 5) was used as a control and was given injections of 250,000 cell/0.1 ml without any peptides. Group two (n = 5) was injected with 250,000 cells and 1000 µg/ml GRGDS in 0.1 ml. Group three (n = 5) was injected with 250,000 cells and 10 µg/ml flavoridin in 0.1 ml. Group four (n = 5) was injected with 250,000 cells and 80 µg/ml flavoridin in 0.1 ml. Group five (n = 5) was injected with 250,000 cells and 50 µg/ml echistatin in 0.1 ml. The puncture was made 4 mm posterior to the limbus in the superonasal quadrant with a 27-gauge needle attached to a tuberculin syringe; care was taken to avoid damage to the lens or contralateral retina. After this, the cells treated with the peptides were injected slowly just above the optic disc under indirect ophthalmoscope. The fundus of each rabbit was examined on days 7, 14, 21, and 28 and localized retinal pucker; grade 1 = tractional retinal detachment involving one medullary ray; grade 2 = tractional retinal detachment involving two medullary rays; and grade 3 = total retinal detachment with a rhegmatogenous component.

**Evaluation of the Retinal Pigment Epithelial Cell Toxicity and Retinal Toxicity**

Cytotoxicity of RPE cells was evaluated with the Alamar blue assay. Two hundred microliters of bovine RPE cells, with a concentration of 100,000 cell/ml, were placed into 96-well tissue culture plates for 24 hours. Flavoridin 10, 20, and 100 µg/ml and echistatin 10, 25, and 50 µg/ml were added to the well. After 24 and 72 hours, 10% Alamar blue diluted with RPMI was added. Alamar blue acts as an oxidation-reduction indicator. Cellular proliferation induced chemical reduction of the dye, which resulted in a color change from blue to red. The intensity of red color, which reflected the extent of cellular proliferation, was determined (at a wavelength of 590 nm) under aseptic conditions by a fluorescence reader (Cytofluor 2300; Millipore, Bedford, MA).

To evaluate the retinal toxicity of the disintegrins, bilateral, simultaneous electroretinograms (ERG) were performed for all animals using an EPIC-2000 unit with a Ganzfeld flash (LKC Technologies; Gaithersburg, MD) on the day before injection and on days 10 and 30 after injection. Rabbits were anesthetized, and one drop of 1% cyclopentolate and 2.5% phenylephrine were instilled into each eye for mydriasis. Scotopic ERGs were performed after at least 30 minutes of dark adaptation with a single Grass flash stimulation, and photopic ERG was performed after at least 5 minutes of light adaptation with a single Grass flash stimulation. To minimize variability, the ratio of the b-wave amplitude of the treated eyes to fellow eyes was used as an index of the retinal function.

Histopathologic examination of the globes of rabbits was used to evaluate the morphologic change after intravitreal injection of disintegrins. On day 30 after treatment, all animals in the experiment were killed by intravenous injection of an excessive dose of pentothal. Globes were enucleated immediately and fixed with phosphated-buffered 10% formaldehyde. The specimens were then dehydrated with increasing concentrations of ethanol, cleared with xylene, and embedded in paraffin. Ten-micron sections were cut and stained with hematoxylin and eosin. Tissue sections were observed and photographed by light microscopy.

**Statistical Analysis**

A Mann–Whitney nonparametric test was used to compare the grading of TRD between the treatment group and the control group in the rabbit model of PVR. An unpaired, two-tailed Student’s t-test was used to compare the results in the cell adhesion assay, vitreous gel contraction assay, and the b-wave amplitude ratio between the treated and control groups.

**RESULTS**

**Integrins Expressed on the Cultured Retinal Pigment Epithelial Cells**

To identify the integrins expressed on the RPE cells, monoclonal antibodies specific to α5, αv, and β1, integrins subunits were used to stain bovine and rabbit
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GRGDS and Disintegrins Inhibit Retinal Pigment Epithelial Cell Attachment to Extracellular Matrix

Figure 2 shows the inhibitory effect of the GRGDS peptide and two disintegrins, flavoridin and echistatin, on the bovine RPE cells attachment to ECM. GRGDS and disintegrins inhibited RPE cell attachment to ECM in a dose-dependent manner. GRGDS inhibited cell binding to fibronectin by approximately 60%. At doses below 150 μg/ml, it inhibited cell attachment to vitronectin by approximately 40%, and, at higher doses, it inhibited cell binding by as much as 90%. At 10 μg/ml, echistatin inhibited cell binding to fibronectin and vitronectin by 70% and 90%, respectively. At 10 μg/ml, flavoridin inhibited cell binding to fibronectin and vitronectin by approximately 30% and inhibited cell binding by approximately 70% at 20 μg/ml. On a molar basis, disintegrins were approximately 150 to 300 times more potent than GRGDS at inhibiting cell adhesion to fibronectin and vitronectin.

Collagen type I and collagen type IV are the predominant types of collagen in interstitial connective tissue and in basement membranes, respectively. GRGDS and the two disintegrins were more potent in inhibiting collagen type I- than type IV-mediated adhesion. The disintegrins were more active than GRGDS at blocking attachment to either substrate. Laminin is the major noncollagenous glycoprotein of basement membrane. GRGDS and disintegrins were less effective in suppressing cell attachment to laminin than to fibronectin, vitronectin, collagen type I, and collagen type IV.

Disintegrin-Inhibited Retinal Pigment Epithelial Cell-Mediated Vitreous Gel Contraction

An in vitro bovine vitreous gel contraction model was used to measure the inhibitory effect of GRGDS and disintegrins. The cell number used was increased 10-fold. Therefore, the dose of the peptides was increased 10-fold as well. GRGDS had no significant inhibitory effect on RPE cell-mediated vitreous gel contraction (Fig. 3A), whereas flavoridin and echistatin inhibited RPE cell-mediated vitreous contraction in a dose-dependent manner. At 80 μg/ml, flavoridin inhibited vitreous contraction to 76% and 74% of its initial volume at 24 and 72 hours, respectively (Fig. 3B). At 50 μg/ml, echistatin inhibited vitreous contraction to 78% and 75% of its initial volume at 24 and 72 hours, respectively (Fig. 3C). Flavoridin and echistatin had the same potency on a molar basis.

Disintegrins Suppressed Retinal Pigment Epithelial Cell-Mediated Tractional Retinal Detachment

The suppressing effect of disintegrins on the RPE cell-mediated TRD was evaluated with a well-established

RPE cells grown on the coverslips. Integrins α2β1 and α5β1 represent the receptors of collagen and fibronectin, respectively, the two major components of proliferative membranes. For bovine RPE cells, it was estimated that more than 80% of the cells were stained positively for integrins α2, α5, and β1. The staining pattern was not homogenous. Instead, there were fluorescence cluster on the cellular surface (Fig. 1A). In some cells, the clusters also were found on the border of the ruffled cell membrane (Fig. 1B).
animal model. In the control group, two eyes developed TRD involving one ray and three eyes had TRD involving two rays at week 2 after surgery. At week 4, one eye had TRD involving two rays and four eyes developed total retinal detachment. When 1000 \( \mu g/ml \) of GRGDS was injected along with RPE cells, five eyes developed TRD (three eyes, grade 1; two eyes, grade 2) at week 2. Five eyes had total retinal detachment at week 4. Table 1 shows the results of the groups treated with 10 \( \mu g/ml \) flavoridin, 80 \( \mu g/ml \) of flavoridin, and 50 \( \mu g/ml \) of echistatin. In the group treated with 10 \( \mu g/ml \) flavoridin, two eyes were grade 0, two eyes were grade 1, and one eye was grade 2 at week 2. However, TRD progressed, and two eyes were grade 1, one eye was grade 2, and two eyes were grade 3 at week 4. Flavoridin at 80 \( \mu g/ml \) and echistatin at 50 \( \mu g/ml \) significantly suppressed the development of the TRD in rabbit eyes.

### Disintegrins Had No Toxic Effect In Vitro and In Vivo

Incubation of bovine RPE cells with flavoridin and echistatin for 24 hours and even 72 hours did not affect proliferation of bovine RPE cell as assessed by the Alamar blue test, a nonisotopic cellular proliferation assay. Figure 4 shows the effect of flavoridin and echistatin on the proliferation of bovine RPE cells. With increasing doses of disintegrins, the proliferation of RPE cells was not affected at either 6 hours or 24 hours after Alamar blue was added. A normal growth efficiency was observed even after the prolonged incubation (more than 72 hours) of flavoridin and echistatin with bovine RPE cells (data not shown).

Electroretinography was used to evaluate the retinal function of rabbit eyes after disintegrin treatment. Both scotopic and photopic amplitude ratios (treated eyes–fellow eyes) of the control group were depressed according to the severity and extent of retinal detachment (data not shown). Figure 5 shows the results of ERGs of the groups treated with flavoridin (80 \( \mu g/ml \)) and echistatin (50 \( \mu g/ml \)) in rabbit eyes. Neither flavoridin nor echistatin perturbed the b-wave amplitude ratios at the 10th day and 30th days after surgery compared to preinjection levels.

Histopathologic examination was performed to check morphologic changes of the retina of rabbit eyes after disintegrin treatment. Figure 6A shows the
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FIGURE 3. Vitreous contraction assay of bovine retinal pigment epithelial cells. (A) GRGDS peptide, (B) flavoridin, (C) echistatin. Data represent means of quadruplicate assays. Student’s t-test; *P < 0.05.

microsection of a rabbit eye treated with flavoridin (80 μg/ml). The eye was enucleated 30 days later. Histologically, the retina maintained normal architecture. A few mononuclear cells were found, reflecting the minimal inflammatory reaction within the eye. The histologic picture of the ciliary body is shown in Figure 6B. The epithelium and stroma of ciliary body were normal in appearance with scanty inflammatory cells. The same condition was found with eyes treated with echistatin (50 μg/ml).

DISCUSSION

Cell-mediated TRD is the fundamental event involved in the pathogenesis of PVR. Basically, it is an aberrant wound healing process, similar to that which occurs elsewhere in the human body. 31 Adhesion, migration, and proliferation of cells, as well as synthesis and secretion of ECMs, are two major components of this process. Integrins, a group of cell surface ECM receptors, play an important role in the interaction between cells and ECMs by transducing biochemical signals from ECMs to the interior of the cells. 32 In this study, indirect immunofluorescent stain demonstrated that integrins formed the adhesion plaques on the surface of the well-attached bovine RPE cells and on the ruffled membrane of the migrating cells. This is consistent with the findings of previous studies. 3334 Although only anti-α1, α2, and α5 subunit monoclonal antibodies were used in the experiment, we think both bovine and rabbit RPE cells may express integrins of β1 family and α5β3 integrin on their surface. The presence of α2β1 and α5β3 integrins in RPE cells may facilitate their attachment to collagen, fibronectin, vitronectin, and laminin. Only when RPE cells establish attachment can migration and proliferation occur. The cell-binding domain of ECM has been determined, and it consists of an RGD amino acid sequence. Avery found that a synthetic tetrapeptide, Arg-Gly-Asp-Ser (RGDS), inhibited RPE cell attachment to fibronectin, collagen type I, and type II in a dose-dependent manner. Because RPE cells cannot survive long if attachment to an appropriate substrate is prevented, it is reasonable to predict that anti-adhesion therapy to the RPE cells will be promising in the management of PVR.

Disintegrins, a group of RGD-containing peptides from some viper venoms, seem to be a potential candidate for the anti-adhesion therapy. These proteins are
TABLE 1. Degree of Retinal Detachment After Disintegrins Treatment

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P value

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*Mann-Whitney test.

PVR = proliferative retinopathy.
PVR grade 0 = normal retina with or without pucker; 1 = tractional retinal detachment involving one ray; 2 = tractional retinal detachment involving two rays; 3 = total retinal detachment with break.

Potent inhibitors of platelet aggregation, which is mediated by fibrinogen binding to the fibrinogen receptor (glycoprotein IIb–IIIa complex) present on the surface of platelets,28 The potential therapeutic usefulness of interfering with integrin-mediated events has been demonstrated in vivo by inhibition of platelet-dependent coronary thrombus formation in canines,23 prevention of the loss of platelets in the extracorporeal circulation of human blood,20 prevention of osteoporosis by inhibiting bone resorption,39 and suppression of metastasis of melanoma cells.26

Echistatin and flavoridin were used in this experiment. Their inhibitory effect on the RPE cell attachment to ECM was evaluated by the cell adhesion assay. On a molar basis, disintegrins displayed much higher potency—150 to 300 times higher—than that of GRGDS peptide. Echistatin inhibited cell attachment to fibronectin and vitronectin more effectively than did flavoridin, whereas flavoridin inhibited cell attachment to collagen more effectively. Both peptides showed the least inhibitory effect on cell attachment to laminin. Because the RGD sequence is found in the macromolecules of ECM, such as fibronectin, collagen, vitronectin, and laminin, the difference of inhibitory effect toward ECM is probably caused by the different RGD configuration of different disintegrins. Echistatin contains 49 amino acids with eight cysteine residues,21 and flavoridin contains 70 amino acids with 12 cysteine residues.22 It is thought that the RGD sequence is the integrin recognition site of disintegrins. However, the potency of these peptides in inhibiting integrin–ligand interaction probably is dependent on both the specific conformation of the RGD sequence determined by the disulfide linkages and the amino acids adjacent to it. This could explain why disintegrins have a higher potency than the linear GRGDS pentapeptide.

The release of viable RPE cells into the vitreous cavity appears to be the initial stage of formation of PVR.40 Once RPE cells contact vitreous, they change their morphology and biologic behavior and become fibroblast-like cells.41,42 Vitreous gel contains type II collagen as its principal fibrillar collagen, as well as type V, IX, and XI collagen, hyaluronic acid, and fibronectin.55,44 Thus, to mimic the more complicated environment in the formation of PVR, the bovine vitreous gel contraction assay was used. When RPE cells were preincubated with disintegrins, the vitreous contraction effect of RPE cells was inhibited significantly in a dose-dependent manner. Echistatin and flavoridin showed the same potency on a molar basis. Because the vitreous gel consists of multiple components and because RPE cells can express various types
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FIGURE 5. The b-wave amplitude ratios of electroretinograms (ERGs) of rabbit eyes after intravitreal injection of (A) flavoridin and (B) echistatin. Values represent mean ± SD. Open bar = scotopic ERG; striped bar = photopic ERG.

of integrins on their surfaces, more than one mechanism might be involved in the inhibitory effect of disintegrins. The RGD-dependent interference on the integrin-ligand binding is probably still the major mechanism. However, a non-RGD-recognition mechanism may play some active role in inhibiting RPE cell binding to collagen. Kupper found that human RPE cells can cause contraction of bovine vitreous, and this activity is mediated principally by αβ integrin, a collagen receptor. The effect can be blocked by another synthetic peptide, ADGAE, a specific collagen-receptor recognition sequence. Because of the complexity of the secondary and tertiary structure of disintegrins, both RGD-dependent and non-RGD-dependent mechanisms might be involved actively in the inhibitory effect of disintegrins on vitreous gel contraction.

The actual sequence of events of action of disintegrins in inhibiting RPE cell-mediated TRD is unknown. Disintegrins are apparently nontoxic to RPE cells because cell proliferation is not altered by adding disintegrins to the medium of cultured cells for 24 and 72 hours. Furthermore, in the experiment of platelet aggregation, flavoridin was found to inhibit specifically fibrinogen binding to fibrinogen receptors on the platelet surface without any inhibitory effect on the intracellular events of the platelet activation pro-

FIGURE 6. Histologic appearance of (A) retina and (B) ciliary body of the eye 30 days after flavoridin treatment. Hematoxylin-eosin stain; magnification, ×250.

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Therefore, the inhibitory effect cannot be explained simply by the cytotoxicity of disintegrins. Results of the animal experiment showed that a low dose of flavoridin (10 μg/ml) did delay the development of TRD in the second week but could not prevent the formation of TRD in the 4th week. The development of TRD could be inhibited only by large doses of disintegrins, e.g., 80 μg/ml flavoridin and 50 μg/ml echistatin. Accordingly, we could deduce that disintegrins serve as reversible antagonists that compete with macromolecules of ECM to bind to the integrin receptors on the surface of RPE cells. At lower doses of disintegrins, the integrin receptors were not occupied fully by disintegrins. Therefore, disintegrins delayed, but did not prevent, the development of TRD. When the receptors were occupied fully by disintegrins, the attachment of RPE cells to the ECM was prevented. If the prevention of attachment lasted longer, RPE cells would not have survived in the vitreous, and TRD would not have developed. However, every experiment has its flaw. In this experimental model, disintegrins were injected simultaneously with RPE cells. In actual human conditions, the proliferation of RPE cells may happen simultaneously with retinal tear or detachment. The attachment of RPE cells to the retina and vitreous was established before the PVR was recognized clinically. It would not be possible to administer a disintegrin at this time. Refinement of the experimental model imitating the actual clinical conditions is needed for further investigation of the role of disintegrins in inhibiting TRD.

The inhibitory effect of disintegrins has been reported to be transient and to have a short half-life. It was found that the inhibitory effect of echistatin on platelet aggregation and bleeding time were reversible 30 to 60 minutes after termination of the infusion in the canine model of thrombosis formation.²³ Cook et al²⁵ found that radiolabeled trigramin, an analog of flavoridin, has a short half-life in the hamster circulation. The half-life of disintegrins in the vitreous has not yet been determined. Because the vitreous cavity is a closed system with a slow turnover rate of aqueous compared with the fast circulation of the bloodstream, one can predict that their actions will last longer in the vitreous cavity. Degradation or immunologic reaction might play some role in the elimination of the foreign disintegrins from the vitreous cavity. More studies will be performed to investigate the pharmacokinetics of disintegrin peptides in the vitreous cavity.

Histology and ERG response demonstrated that disintegrins had no toxic effect on rabbit retina when they were introduced into the vitreous cavity. Moderate inflammatory reaction could be found in the first 2 weeks after injection. However, there was no significant difference in inflammation between the disintegrin injection and control groups. In addition, there was no adverse side effect, such as hemorrhage, observed with disintegrin-injected eyes. Therefore, disintegrins seem to be potential candidates for the prevention of cell-mediated TRD. Recently, echistatin has been obtained by chemical synthesis,³⁵ and by recombinant DNA technology.⁴⁰,⁴⁰ It provides a powerful tool for basic and clinical research of cell–ECM interaction. Meanwhile, the sequence of a family of cellular proteins related to snake venom disintegrins has been deduced from the cDNA sequence from various mouse tissues and a mouse cell line.⁵¹ Characterization of this disintegrin superfamily will be helpful for further understanding the cell–cell and cell–matrix interactions under physiological conditions and some pathologic disorders, such as PVR.

In conclusion, this study demonstrates the effectiveness of disintegrins, flavoridin, and echistatin, as inhibitors of RPE cell-mediated TRD. Disintegrins and their analogs have potential usefulness as an anti-adhesion therapeutic agents in various ocular disorders in which the inhibition of integrin function is desired. However, further studies are required to determine the feasibility of this approach for other ocular disorders associated with abnormal wound healing, such as PVR or glaucoma filtering surgery.

**Key Words**

disintegrin, extracellular matrix, integrin, proliferative vitreoretinopathy, retinal pigment epithelial cell

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