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Effects of mechanical stress and vitreous samples in retinal pigment epithelial cells

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ABSTRACT

In rhegmatogenous retinal detachment (RRD), scattered RPE cells from the basement membrane into the vitreous cavity undergo an epithelial mesenchymal transition (EMT) and form the intraocular fibrous membrane in response to vitreous fluid.

We investigated whether exposure to vitreous samples was associated with EMT-associated signals and mesenchymal characters. Human vitreous samples were collected from patients with RRD, epiretinal membrane (ERM), or macular hole (MH). We evaluated the effects of vitreous on ARPE-19 cells in suspension cultures using poly 2-hydroxyethyl methacrylate-coated dishes and three-dimensional (3D) Matrigel cultures. We found that exposure to vitreous samples did not induce morphological changes or accelerate wound closure in monolayers. Several samples showed increased phosphorylation of Smad2 and nuclear translocation of nuclear factor-κB. Mechanical stress triggered an elevation of phosphorylation levels in Smad2. In addition, exposure to vitreous fluid increased the phosphorylation of p38 mitogen-activated protein kinase in cell suspension cultures after mechanical stress. Moreover, ARPE-19 cells showed a stellate invasive phenotype in 3D Matrigel cultures with vitreous samples.

In this study, we demonstrated that mechanical stress and vitreous were associated with EMT-associated signals and invasive phenotypes in 3D cultures but not in monolayers. These results have important implications for the role of vitreous humor in the induction of EMT and intraocular fibrosis.

1. Introduction

In the eye, retinal pigment epithelial (RPE) cells have been regarded as important components of intraocular fibrotic changes that occur during proliferative vitreoretinopathy (PVR) and age-related macular degeneration (AMD) [1–3]. For example, Anderson et al. reported that the dedifferentiation and proliferation of RPE cells occurred after rhegmatogenous retinal detachment (RRD) [4]. Some investigators hypothesized that chemical factors contained in the vitreous fluid could be mitogenic to RPE cells [4]. This hypothesis was supported by the observation that vitreous aspirates from patients with PVR stimulated the migration of RPE cells [5], and vitreous fluid had an effect on cell proliferation [6], which appeared to be mediated through TGF-β [7]. However, since another investigation demonstrated differential effects from TGF-β and vitreous fluid on the transformation of RPE cells [7], it is considered likely that TGF-β and other factors might cooperatively induce fibrosis arising from RPE cells.

Conversely, recent investigations revealed that an aberrant epithelial–mesenchymal transition (EMT) is involved in the development of fibrotic disorders and cancer invasion [8]. We previously reported that the co-stimulation with tumor necrosis factor-α (TNF-α) and TGF-β induced EMT by promoting hyaluronan-CD44 interactions in RPE cells (ARPE-19) [9]. It has been reported that there are high levels of TGF-β, specifically in vitreous specimens from subjects with PVR [10]. In addition, impairment of the blood-retinal barrier has been shown to cause increased levels of TNF-α in inflammatory disorders [11]. Therefore, we hypothesized that RPE cell exposure to vitreous fluid is an important step in the onset of EMT signaling, leading to fibrotic changes associated with vitreoretinal disorders after retinal detachment.

In this study, we reported that exposure of adherent RPE cells to vitreous fluid was not sufficient to activate the EMT signaling pathway. However, additional mechanical detachment of the RPE

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epithelial monolayer induced Smad phosphorylation, and subsequent exposure to vitreous promoted p38 mitogen-activated protein kinase (MAPK) activation. In addition, vitreous fluid led to mesenchymal responses in three-dimensional (3D) Matrigel cultures.

2. Materials and methods

2.1. Human vitreous samples

Human vitreous samples were obtained during vitrectomies performed on patients with RRD, epiretinal membranes (ERM) or a macular hole (MH). The samples were undiluted by manual suction into a 2.5 ml syringe through an air-flushed aspiration line with a vitreous cutter before opening the infusion line. These experiments were performed in accordance with the tenets of the Declaration of Helsinki. Vitreous samples were collected after obtaining written informed consent and approval by the Ethics Committee at Kumamoto University.

2.2. Cell culture

ARPE-19 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium-nutrient mixture F-12 (Sigma) supplemented with 10% fetal bovine serum.

2.3. Reagents and antibodies

Human recombinant TNF-α and human recombinant TGF-β_2 were obtained from R&D Systems (Minneapolis, MN, USA). Antibodies for anti-phospho-Smad2, anti-Smad2, anti-phospho-nuclear factor-κB (NF-κB), anti-phospho-p38 MAPK, and anti-p38 MAPK were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-NF-κB and anti-N-cadherin antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The anti-CD44 antibody was purchased from BioLegend (San Diego, CA, USA).

2.4. Immunofluorescent microscopy

Immunofluorescent analysis was performed as previously described [12]. In brief, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% TritonX-100 and then incubated with primary antibodies for 60 min at room temperature. After washing, cells were incubated with either an Alexa-488 or Alexa-596-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) for 60 min. Actin filaments were detected by Alexa-488 labeled phalloidin (Molecular Probes). Cells were mounted and viewed with a confocal microscope (FluoView; Olympus, Tokyo, Japan).

2.5. Immunoblot analysis

We used a protocol from a previous study [12]. Briefly, cells were lysed, and protein concentrations were assayed using the BCA assay. Equal protein amounts from the samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). After blocking, membranes were exposed to primary antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies. The immune complexes on the membrane were detected by ECL Western Blotting Detection Reagents (GE Healthcare, Tokyo, Japan).

2.6. Wound healing assay

A confluent monolayer of ARPE-19 cells were incubated with or without 20% vitreous samples in serum-free medium for 24 h at 37 °C and 5% CO_2. A wound was made by scratching using a sterile plastic micropipette tip, and cells were further incubated in serum-free medium with 20% vitreous fluid. Cell migration into the wounded area was photographed by DIC microscopy (Olympus).

2.7. Suspension culture

ARPE-19 cells were detached from the culture dish by scraping with a silicon rubber scraper or treatment with Trypsin–EDTA (Gibco, Grand Island, NY, USA). Detached cells were seeded into poly 2-hydroxyethyl methacrylate (HEMA) (Tokyo Kasei Industry, Tokyo, Japan)-coated dishes to prevent cellular attachment. The cells were cultured in serum-free medium with or without a combination of TNF-α and TGF-β_2 or with 10% human vitreous for 60 min at 37 °C and 5% CO_2. Cells were collected by centrifugation and subjected to immunoblot analysis.

2.8. Three-dimensional (3D) Matrigel culture

After Growth Factor Reduced-Matrigel (BD Lifesciences) polymerization on the chamber slide (Thermo Scientific, Rockford, IL, USA), cells were seeded onto the Matrigel and incubated in medium containing 10% FCS and 2% Growth Factor Reduced-Matrigel, including different concentrations of vitreous samples.

3. Results

3.1. Effects of vitreous stimulation in ARPE-19 cells

Human vitreous samples were surgically obtained from two patients with epiretinal membrane (ERM #1 and ERM #2) and three patients with rhegmatogenous retinal detachment (RRD #1, RRD #2, and RRD #3). In the immunoblot analyses, the additive effects of vitreous samples on cultured ARPE-19 cells were different in each case. The phosphorylation of Smad2 and p38 MAPK was observed in the experiments using vitreous samples from ERM #1, ERM #2, and RRD #3 (Fig. 1A) but not in those from RRD #1 and RRD #2. Although none of the vitreous samples increased the phosphorylation of NF-κB in the immunoblot analysis (Fig. 1A), immunostaining studies showed nuclear translocation of NF-κB after a 24-h treatment with the vitreous fluid from ERM #1 in several ARPE-cells (Fig. 1B). These data indicated that there were no significant differences in the activation of EMT-associated pathways between the ERM and RRD samples.

We further examined the contribution of vitreous fluid to cell migration with the wound healing assay. Fig. 2 shows the effects of 20% vitreous samples on wound closure in ARPE-19 cells. Our statistical analyses showed no significant differences between the experimental groups and controls for wound healing activities (Fig. 2).

3.2. Effects of vitreous exposure in cell suspension cultures

In order to examine the effects of vitreous stimulation, we conducted further experiments using cell suspension cultures. Confluent ARPE-19 cells were detached by scraping or trypsinization, and seeded into HEMA-coated dishes in serum-free medium with or without the combination of TNF-α (10 ng/ml) and TGF-β_2 (5 ng/ml). The immunoblot analysis showed a remarkable decrease in trans-membrane protein CD44 expression in the trypsin-treated cells. There were no apparent differences in the expression of CD44