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Cell-mediated contraction of vitreous explants from chicken embryo: Possibility of screening for therapeutic agents against proliferative vitreoretinal diseases

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Purpose: We aimed to establish a novel screening system for identifying potential therapeutic agents for treating proliferative vitreoretinal diseases (PVDs). In this study, we focused on vitreous explants from chicken embryos and evaluated the usefulness of quantitatively analyzing the effects of potential candidates on cell-mediated vitreous contraction, which leads to blindness in PVDs.

Methods: Vitreous explants were extracted from 19-day-old embryonic chickens and then incubated with retinal Müller cells or endothelial cells to permit cell adhesion. After cell adhesion occurred, we examined the effect of the attached cells on the wet weight of vitreous explants as an index of vitreous contraction. We also performed hematoxylin and eosin staining to characterize the cell morphology on the vitreous surface.

Results: Contraction of the vitreous explants was observed after cell adhesion of not only retinal Müller cells but also endothelial cells. We confirmed the adhesion of these cells on vitreous explants and estimated the number of adherent cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis. The cells on the vitreous surface presented an elongated fibroblast-like phenotype. Integrin was found to be a receptor involved in cell adhesion on the vitreous surface.

Discussion: Our results suggest that vitreous explants from chicken embryos may be novel useful tools for screening antiadhesion therapeutic agents in PVDs. This preliminary study must be validated with human vitreous and human retinal pigment epithelial cells.

Proliferative vitreoretinal diseases (PVDs) such as proliferative diabetic retinopathy (PDR) and proliferative vitreous retinopathy (PVR) are common causes of severe vision loss [1]. One of the critical causes of blindness by PVDs is tractional retinal detachment caused by the formation of contractile preretinal fibrous membranes. The proliferative membrane then extends into the vitreous humor [2-6], causing tractional retinal detachment. The proliferative membrane consists of various cells [7-12], including endothelial cells, hyalocytes, retinal pigment epithelial (RPE) cells, retinal Müller cells, and fibroblast-like cells. Since it has been reported that the contractile forces on vitreous, which lead to tractional retinal detachment, are cell-mediated [13], antiadhesion therapy is thought to be a strategy for treating and preventing PVDs [14,15].

For the drug screening for therapeutic medicine development, establishing a screening system is indispensable. Contraction analysis with collagen gel is widely used to assess the beneficial effects of drug candidate compounds for treating and preventing PVDs [16,17]. This method is excellent for quantitative analyses. However, it is quite unusual for an experimental system using vitreous explants to mimic the more complicated biologic environment, with the exception of bovine vitreous [18-20].

Vitreous explants from chickens contain the same components [21-25] as the human vitreous. For example, human and chicken vitreous explants have type II collagen (a principal fibrillar collagen), type V collagen, type IX collagen, type XI collagen, and fibronectin [26]. A notable exception is hyaluronic acid, which is replaced in the chicken vitreous by a highly glycosylated version of collagen IX [27]. In addition, since the structure of chicken vitreous explants, especially from chicken embryos, is maintained by a three-dimensional network of collagen fibrils, the quantitative analysis may be applicable. In this study, we used the readily accessible vitreous explants from chicken embryos to establish a novel screening system for identifying potential candidates with antiadhesive action on vitreous explants. We demonstrated that our system could be used to quantitatively assess the number of adhesive cells on the vitreous surface and to observe the cell morphology after cell adhesion. In addition, we estimated the percentage of vitreous contraction

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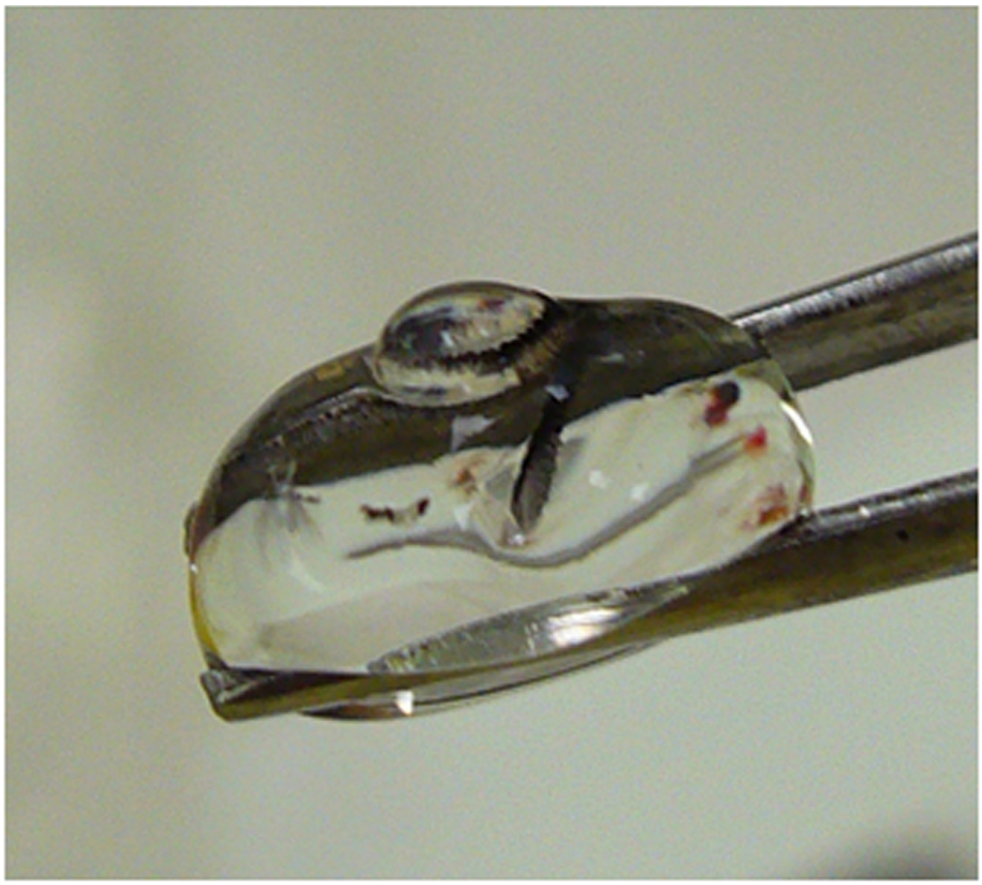

Figure 1. Vitreous explants from chicken embryos. The vitreous explants were prepared as described in the Materials and Methods section. The vitreous explants retained mechanical integrity during experiments and maintained the wet weight, which was measured as an index of mechanical integrity.

by measuring the wet weight as an index of contraction, after cell adhesion. Our results suggest that vitreous explants from chicken embryos may serve as novel tools for screening therapeutic medicines for PVDs.

METHODS

Cell cultures: The human retinal Müller cell line (MIO-M1) was a kind gift from Dr. G. Astrid Limb (UCL Institute of Ophthalmology, London, UK) [28]. Mouse cerebral microvascular endothelial cells (bEnd.3) were obtained from American Type Culture Collection (ATCC; Manassas, VA). Both cell types were cultured in high (25 mM) glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Biowest, Nuaille, France), GlutaMAX-I (Invitrogen), and 1% penicillin-streptomycin-neomycin (PSN) antibiotic mixture (Invitrogen) at 37 °C in a humidified 5% CO_2 atmosphere, until confluency was achieved.

Preparation of vitreous explants: Chicken embryos were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Chicken eyes were obtained from 19-embryonic-day-old (ED) chickens. The anterior segment (cornea) with attached vitreous explants of the eye was removed, and intact vitreous explants (Figure 1) were dissected from the retina. The vitreous explants were dialyzed for at least 30 min against serum-free DMEM. The vitreous explants retained mechanical integrity during this stage. As shown in Table 1, the vitreous explants from the chicken embryos maintained the wet weight, which was measured as an index of mechanical integrity with a validity of at least 24 h.

Cell culture on vitreous surface: The confluent cells were removed from their culture dishes by using 0.05% trypsin and 0.53% EDTA. Thereafter, they were mixed with DMEM containing 10% FBS and centrifuged for 5 min at 240 × *g*. After the supernatant was discarded, the cell pellet was resuspended in DMEM. Aliquots (3 mL) of MIO-M1 cells or bEnd.3 cells, with a cell density of 100,000 cells/mL were applied to the surface of the vitreous explants from the chicken embryos in a conical tube and then incubated at 37 °C with gentle rotation for 2 h to permit cell adhesion. Through the process of cell culture on vitreous surface, we cultured

Table 1. Wet weight of vitreous explants from chicken embryo after temporal incubation.

Incubation time (h)	Wet weight (mg)
0	217.0±6.2 (n=4)
12	220.4±8.0 (n=4)
24	206.1±9.2 (n=4)
48	176.9±16.0 (n=4)
72	153.2±10.3 (n=4)
96	121.6±12.2 (n=4)

the cells in the high glucose condition since there is a report that high glucose is actively involved in cell-matrix adhesion in diabetic retinopathy [29].

Evaluation of cell morphology on vitreous explants: After cell adhesion occurred, vitreous explants were washed with DMEM and fixed in SUPERFIX (KURABO, Osaka, Japan) for 24 h at 4 °C. Thereafter, the vitreous explants were divided into two pieces (based on the proximity to the lens and the retina) for subsequent analysis of the cell morphology on the vitreous surface. This was followed by hematoxylin and eosin (H&E) staining for analysis with a stereoscopic microscope throughout the duration of the experiments.

Quantitative evaluation of adherent cells on vitreous explants: The number of adherent cells was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis. Briefly, the vitreous explants were washed with DMEM to remove non-adherent cells and transferred to a 48-well plate immediately after adhesion on vitreous explants. Fresh medium (500 µl) containing 50 µl MTT (Dojindo Laboratories, Kumamoto, Japan) was added to each well and incubated at 37 °C for 6 h. Absorbance was measured at 450 nm using the Multi-Detection Microplate Reader (DS Pharma Biomedical, Osaka, Japan).

Contraction analysis: After the cell culture on vitreous explants step, the vitreous explants and DMEM (with or without 10% FBS) were mixed and placed in a 24-well plate and incubated at 37 °C for up to 24 h. Macroscopic effects of cell adhesion on vitreous explants were observed in 24-well plates after incubation at 37 °C. For visualization, vitreous explants were stained with Coomassie brilliant blue (CBB) R-250 for 1 min, and washed with PBS containing 2.68 mM potassium chloride, 1.47 mM potassium phosphate monobasic, 136.89 mM sodium chloride and 8.1 mM sodium phosphate dibasic as previously reported [18]. After staining, the morphology of vitreous explants was observed under a stereoscopic microscope. Control vitreous explants without cells were incubated identically. In some vitreous explants, such as porcine or chicken, the wet weight can be measured as previously reported [30] or as shown in Table 1, respectively. For quantitative analysis of vitreous explants, Matsui et al. [30] calculated the percentage of vitreous liquefaction based on the reduction in the wet weight of porcine vitreous explants. With the help of their report, the reduction in the wet weight of vitreous explants after vitreous contraction was evaluated at frequent intervals for up to 24 h after incubation, and the percentage was calculated as follows: Vitreous contraction (%)=[1 - cell-treated vitreous wet weight (mg)/ cell-free vitreous wet weight (mg)] × 100.

Statistical analysis: Statistical significance was calculated using a double-sided unpaired Student *t* test. Significance was calculated from four independent experiments.

RESULTS

Contraction of vitreous explants from chicken embryos by cell adhesion: Several cell types, including retinal Müller cells, are involved in generating tractional forces on the vitreous surface in the pathogenesis of PVDs. First, to assess the ability of Müller cells to generate tractional forces, the retinal Müller cell line, MIO-M1, was placed on the surface of the vitreous explants from the chicken embryos. After incubation for 2 h to permit cell adhesion on vitreous explants, the vitreous explants were transferred to 24-well plates with DMEM (with or without 10% FBS). Changes in the wet weight of the vitreous explants were then measured as an index of contraction for up to 24 h. As shown in Figure 2A, contraction of vitreous explants was measurable within 6 h of culture with 100,000 cells per vitreous explant, and it continued to advance throughout the incubation. The percentage of contraction estimated from alteration in the wet weight of the vitreous was 32.1%, 53.6%, and 71.4% at 6 h, 12 h, and 24 h, respectively ($p<0.05$). Although the percentage of contraction tended to increase in the presence of FBS, a known source of contraction-promoter at the stage of cell culture on the vitreous surface in cell model system [31], there was no statistically significant difference in our studies. In

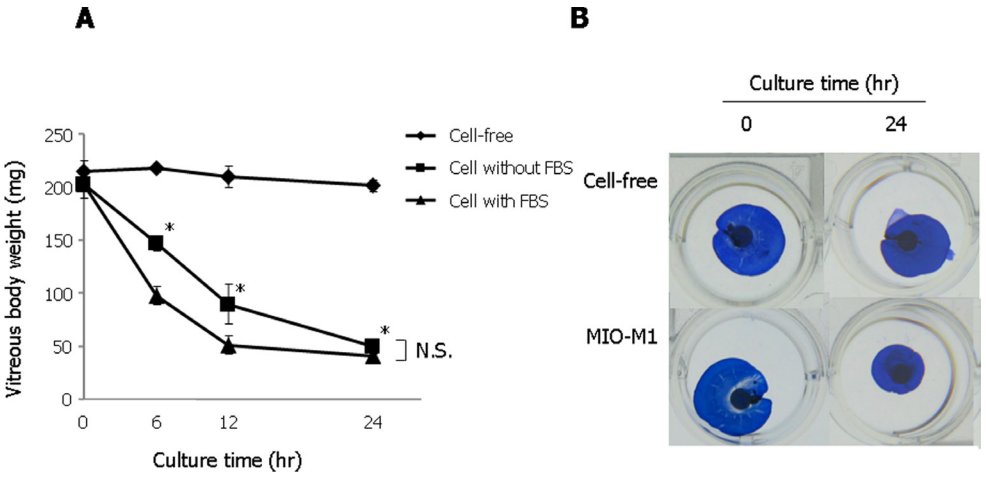

Figure 2. Vitreous contraction analysis of retinal Müller cells in vitreous explants from chicken embryos. **A**: After cell adhesion of human retinal Müller cell line (MIO-M1) cells, the vitreous explants were incubated in Dulbecco's modified Eagle's medium (DMEM), in the presence or absence of fetal bovine serum (FBS), at 37 °C up to the indicated periods. The wet weight of vitreous explants was measured for the quantitative analysis of vitreous contraction. Control vitreous explants were incubated at 37 °C in the culture medium for 24 h in the absence of cells. **B**: Vitreous explants were cultured with adherent MIO-M1 cells under the same conditions. Thereafter, the vitreous explants were stained with Coomassie brilliant blue (CBB) to evaluate the macroscopic effects of cell adhesion. Data are expressed as mean±standard deviation (SD; n=4). *, significantly different from cell-free control group ($p<0.05$). Not significant versus cell without FBS.

addition, contraction of vitreous explants after cell adhesion was also confirmed by staining with CBB (Figure 2B).

We then investigated whether endothelial cells, which are reported to be the other cell type involved in vitreous contraction, could mediate the contraction of vitreous explants from the chicken embryo. As shown in Figure 3A, an endothelial cell line, bEnd.3, also induced a substantial contraction, producing a measurable reduction in the wet weight of vitreous explants within 6 h and achieving 42.6% reduction in the wet weight of vitreous explants within 24 h ($p<0.05$). In a separate assay, the presence of FBS had no effect on either the bEnd.3-induced vitreous contraction or the MIO-M1 cells. In addition, we observed the bEnd.3-mediated vitreous contraction with the CBB staining method (Figure 3B).

Cell morphology on vitreous explants: Next, we examined the cell morphologies on the vitreous surface immediately or 6 h after cell adhesion with H&E staining. As shown in Figure 4, we confirmed the cell adhesion of MIO-M1 cells at the anterior and posterior regions of the vitreous, and the cell morphologies at both regions of the vitreous remained spherical shortly after cell adhesion, and then it became elongated on the surface of vitreous explants at 6 h after

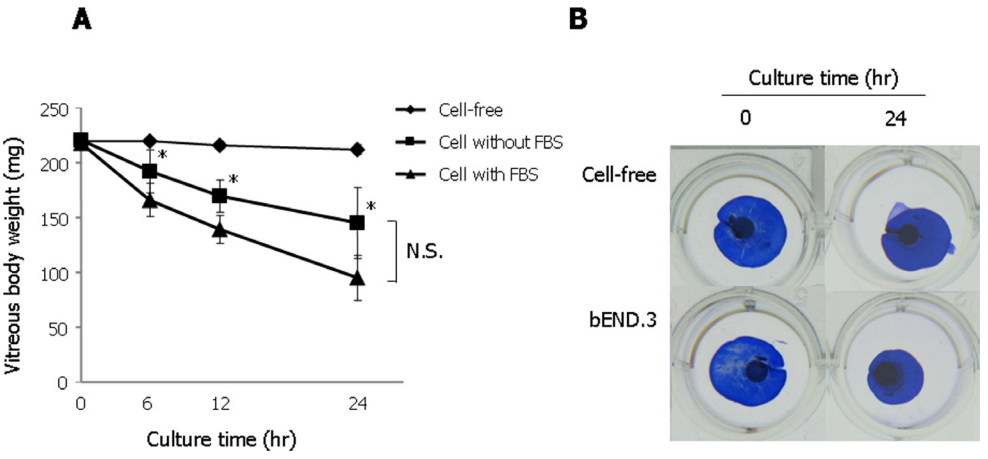

Figure 3. Vitreous contraction analysis of endothelial cells in vitreous explants from chicken embryos. **A**: After cell adhesion of bEnd.3 cells, the vitreous explants were incubated in Dulbecco's modified Eagle's medium (DMEM), in the presence or absence of fetal bovine serum (FBS), at 37 °C up to the indicated periods. Thereafter, the wet weight of vitreous explants was measured for the quantitative analysis of vitreous contraction. Control vitreous explants were incubated at 37 °C in the culture medium for 24 h in the absence of cells. **B**: Vitreous explants were cultured with adherent bEnd.3 cells under the same conditions. Thereafter, the vitreous explants were stained with Coomassie brilliant blue (CBB) to evaluate the macroscopic effects of cell adhesion. Data are expressed as mean±standard deviation (SD; n=4). *, significantly different from cell-free control group ($p<0.05$). Not significant versus cell without FBS.

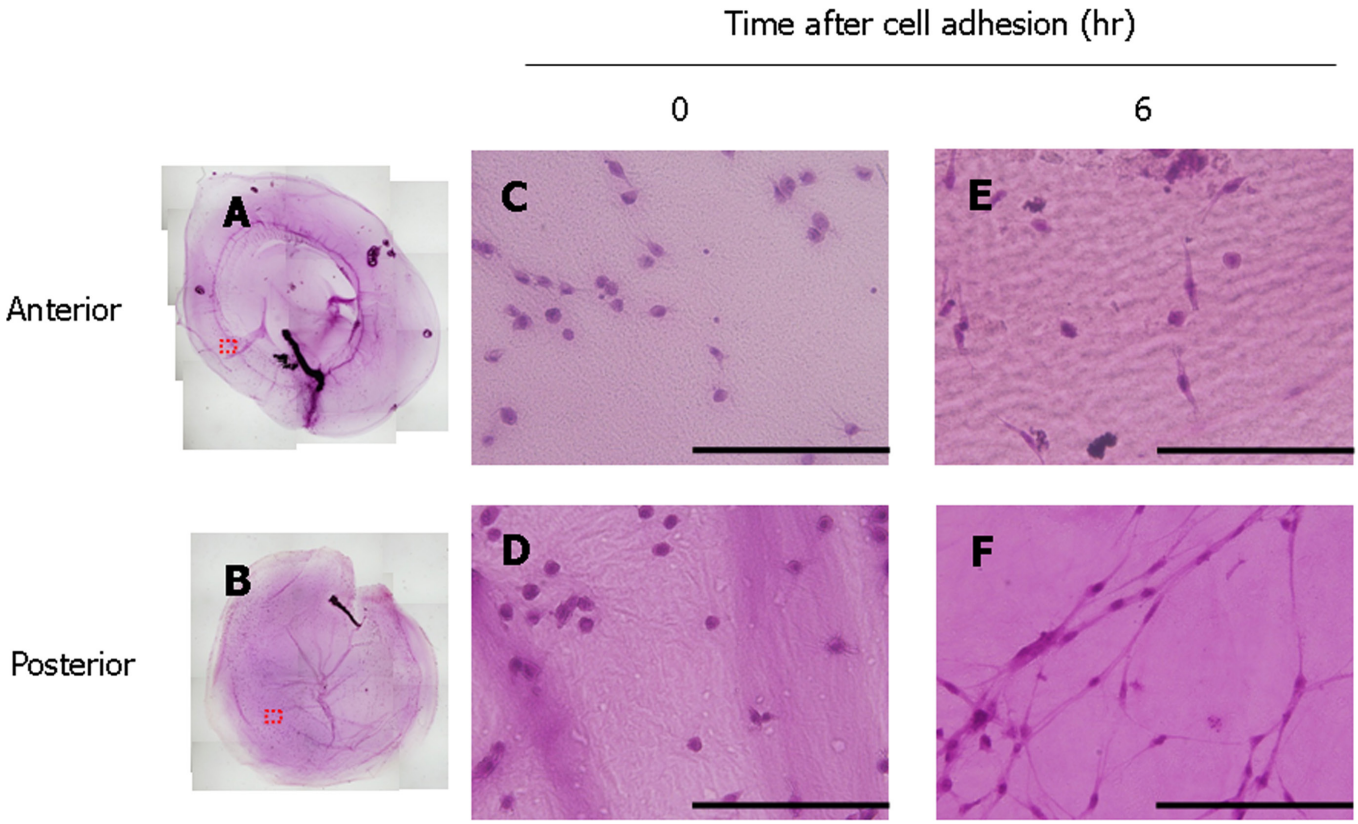

Figure 4. Morphologies of retinal Müller cells contracting vitreous explants from chicken embryos. Stereomicroscopic images of human retinal Müller cell line (MIO-M1) cells on the vitreous surface were taken after hematoxylin and eosin (H&E) staining at the indicated periods. The micrograph depicts cell morphology on the vitreous surface at the anterior (**A**) or the posterior region (**B**) and the magnified image is shown in (**C**) or (**D**), respectively. The magnified image (**E** or **F**) is the cell morphology on the vitreous surface at the anterior or the posterior region, respectively, at 6 h after cell adhesion. Scale bar=200 µm.

cell adhesion. Müller cells located on the posterior region of vitreous explants notably present an elongated fibroblast-like phenotype. Similar to Müller cells, bEnd.3 cells spread out on the vitreous surface, and almost all the cells had a spindle-shaped morphology at 6 h after cell adhesion, regardless of whether they originated from the anterior or the posterior region of the vitreous (Figure 5).

Effect of EDTA and heparin on cell adhesion and vitreous contraction: To determine the cellular receptors involved in adhesion, we evaluated the effect of EDTA and heparin on the cell attachment of MIO-M1 or bEnd.3 cells to vitreous explants. EDTA inhibits integrin-mediated cell adhesion, which requires divalent cations for activation. However, heparin inhibits cell adhesion via syndecans, a membrane associated with heparan sulfate proteoglycans. When EDTA was preincubated with MIO-M1 cell suspension for 2 h and then added to the vitreous explants, attachment of MIO-M1 cells to the anterior and posterior regions of vitreous explants was completely inhibited (Figure 6A). In contrast, heparin had little effect on cell adhesion to vitreous surface. We further quantitatively evaluated the number of adherent cells on vitreous explants with MTT analysis. The results concurred with the reported data, and thus, the number of MIO-M1 cells adhered to whole vitreous explants markedly decreased in EDTA-pretreated vitreous explants ($p<0.05$; Figure 6B). Heparin data were also corroborated with MTT analysis, which showed that heparin treatment had no effect on the number of adherent MIO-M1 cells. Similar results were obtained using bEnd.3 cells (Figure 7). Taken together, these data suggest that the vitreous contraction may be a cell-mediated process involving functional integrin, but not heparan sulfate proteoglycans.

Finally, we investigated the effect of EDTA and heparin on vitreous contraction. As expected, pretreatment with EDTA significantly prevented the cell-mediated vitreous contraction (Figure 8) in the MIO-M1 ($p<0.001$) and bEnd.3 ($p<0.01$) cells. However, when MIO-M1 or bEnd.3 cells were

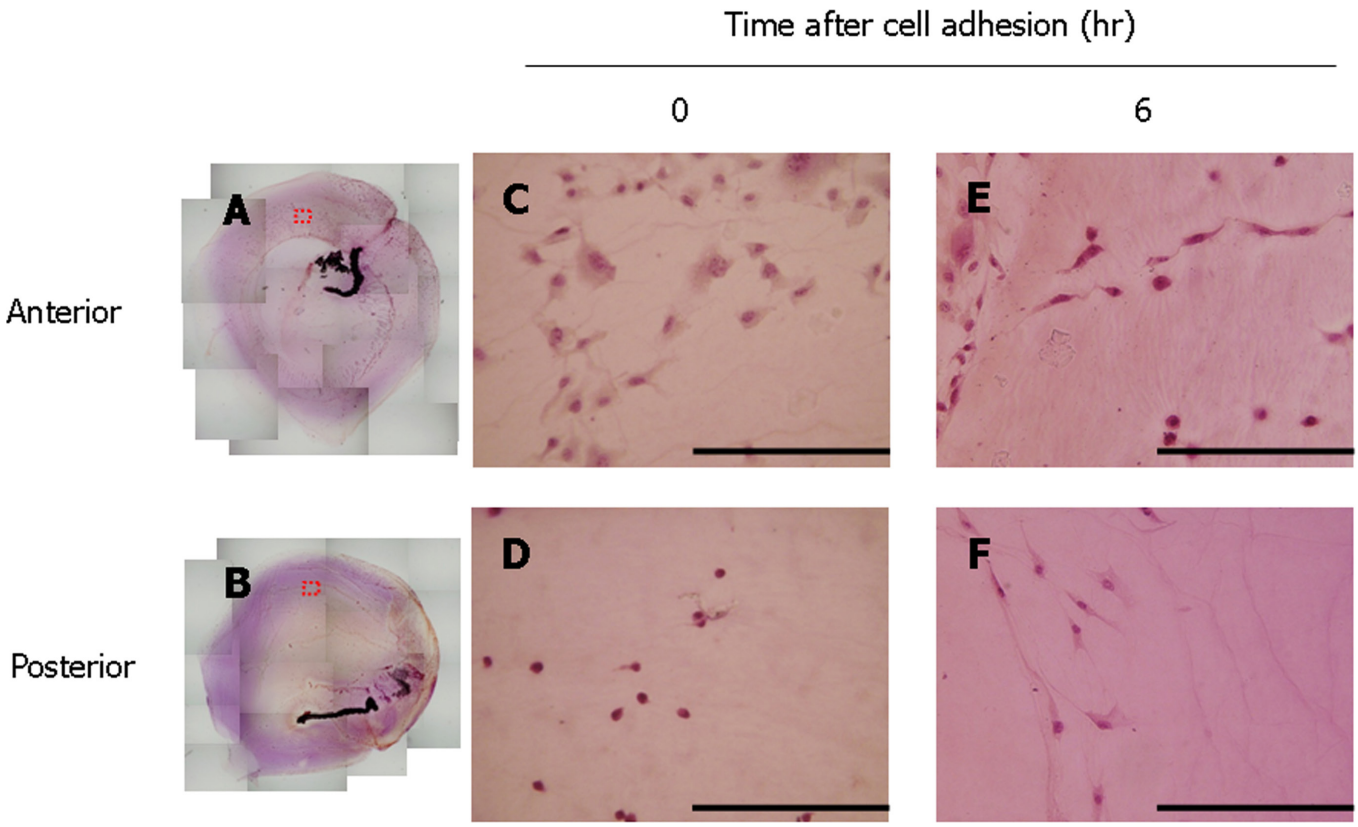

Figure 5. Morphologies of endothelial cells contracting vitreous explants from chicken embryos. Stereomicroscopic images of bEnd.3 cells on vitreous surface were taken after hematoxylin and eosin (H&E) staining at the indicated periods. The micrograph depicts cell morphology on the vitreous surface at the anterior (**A**) or the posterior region (**B**) and the magnified image is shown in (**C**) or (**D**), respectively. The magnified image (**E** or **F**) is the cell morphology on the vitreous surface at the anterior or the posterior region, respectively, at 6 h after cell adhesion. Scale bar=200 µm.

preincubated with heparin, there was no significant effect on cell-mediated vitreous contraction.

DISCUSSION

Antiadhesion therapy has been regarded as a promising option for treating and preventing PVDs [14,15]. To conduct drug screening for potential therapeutic medicine, establishing a screening system is indispensable. In the present study, we established a novel quantitative-screening system for enabling the searching of potential candidates with antiadhesive action by using vitreous explants from chicken embryos. Although bovine vitreous explants have been used to evaluate macroscopic changes such as vitreous volume and cell morphology on the vitreous surfaces after cell adhesion [18,19], exhaustive analysis is thought to be restricted by unavailability of a massive amount of bovine vitreous explants. Our system uses readily accessible vitreous explants from chicken embryos, and evaluates the effect of potential compounds on cell-mediated vitreous contraction more efficiently.

Cell-extracellular matrix (ECM) interaction is involved in vitreous contraction in PVDs. The integrins and the syndecans are known to major adhesion receptor families that mediate adhesion in various tissues [32]. Especially, integrins are a class of heterodimeric cell surface ECM receptors, actively involved in generating tractional force in PVDs. Robbins et al. [33] have demonstrated that numerous integrin subunits are expressed prominently by cells in proliferative retinal membranes. In addition, previous studies have demonstrated integrin-dependent collagen contraction by fibroblast [34-36] or RPE cells [37]. Müller cells are also known to generate tractional forces [11,12,38,39]. Guidry et al. [40] compared the ability of porcine choroidal fibroblasts, retinal glial cells that belong to Müller cells, and RPE cells to contract collagen gels, and demonstrated that glial cells are the most effective, followed by choroidal fibroblasts and RPE cells. In addition, Müller cells are reported to express all integrin subunits that comprise the collagen-binding receptors [41]. In this respect, Müller cells are consistent with most other matrix contraction-capable cells [34,42]. In the present

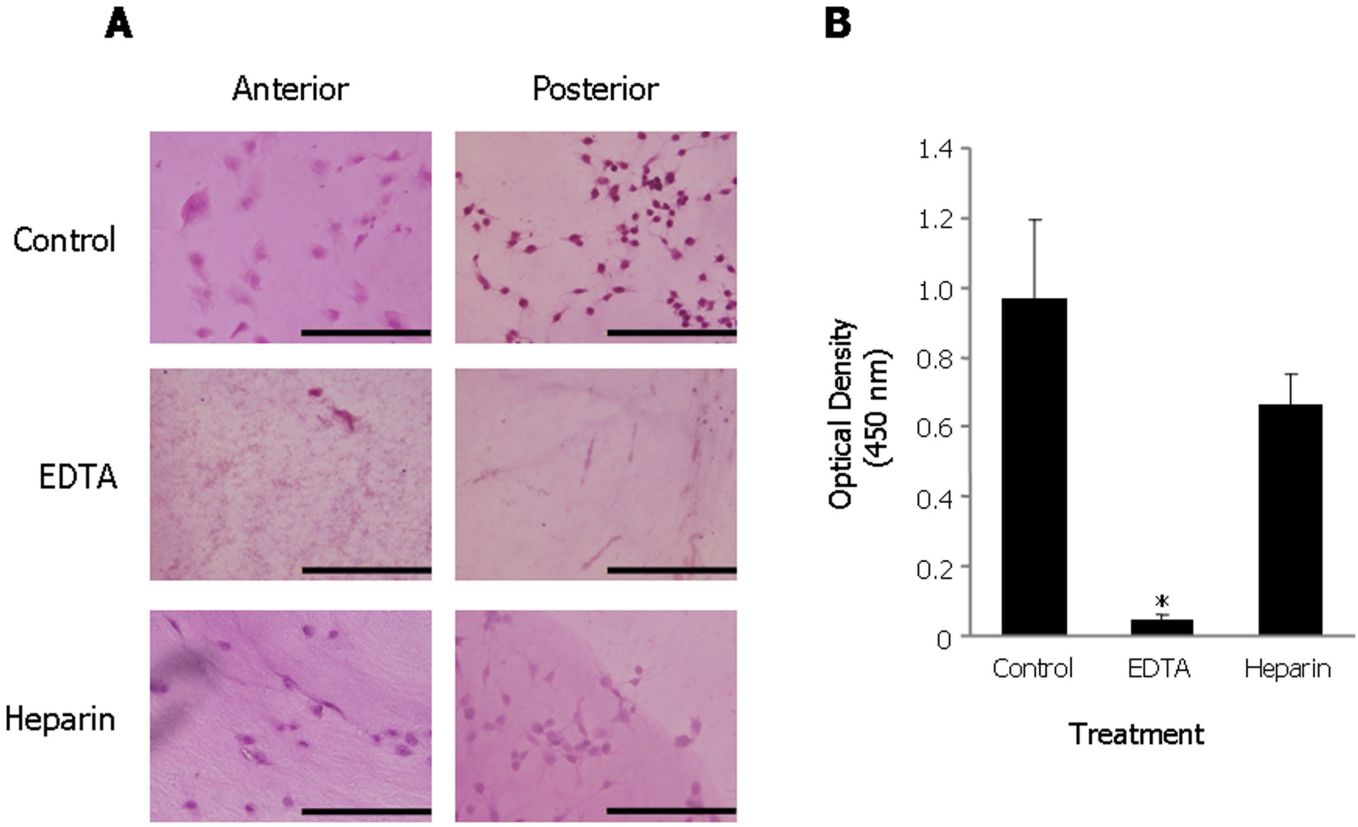

Figure 6. Effect of EDTA and heparin on cell adhesion of retinal Müller cells. **A**: EDTA (EDTA) or EDTA (5 mM) or heparin (10 µg/ml) was preincubated with human retinal Müller cell line (MIO-M1) cell suspension for 2 h and then added to the vitreous explants. Thereafter, stereomicroscopic images of MIO-M1 cells on the vitreous surface were taken after hematoxylin and eosin (H&E) staining. The micrograph depicts cell morphology on the vitreous surface in the magnified image. Scale bar=200 µm. **B**: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the number of adherent cells on the vitreous surface. Data are expressed as mean±standard deviation (SD; n=4). *, significantly different from the cell-treated group ($p<0.05$).

study, Müller cells possessed the contractile activity against vitreous explants from chicken embryos. The contractile activity was abrogated by pretreatment with EDTA, a known integrin inhibitor. These results suggest that the vitreous contraction by Müller cells results in integrin-dependent cell adhesion on vitreous explants. Since the same results were obtained using another endothelial cell line, bEnd.3, which expresses the collagen-binding integrin [43,44], we speculate that it may be possible to use our system for screening of drugs with integrin-dependent antiadherent action. However, heparin had little influence on cell adhesions on vitreous explants from chicken embryos. Therefore, syndecans, a target of heparin, may not participate in vitreous contraction, which arises after cell adhesion.

The temporal cultivation of cells on vitreous explants from chicken embryos caused the morphological changes to fibroblast-like cells. Our results are similar to those available on bovine vitreous explants. Kupper et al. [20] reported that elongated RPE cells are observed on the surface of bovine vitreous explants after temporal cultivation. In addition, vitreous-induced morphological changes were also confirmed in vitro systems [45-47]. Interestingly, Meitinger et al. [45] demonstrated that increased expression of α5 integrin, which is achieved using the adenovirus overexpression system, is sufficient to induce the morphological change. Since several studies have shown that morphological change to fibroblast-like phenotype is closely associated with the ability to generate tractional forces in Müller cells [11,41,48], we speculate that the phenotype change in fibroblast-like cells, which is probably mediated by increased expression of integrin, may be also involved in the contraction of vitreous explants from chicken embryos after cell adhesion. Previous studies [49-52] have indicated that among the integrin subunits, the collagen-binding integrin subunits such as α2 and β1 are components of the receptor involved in transmitting tractional forces generated by human Müller cells. Further investigation is needed to address whether these integrin subunits are also

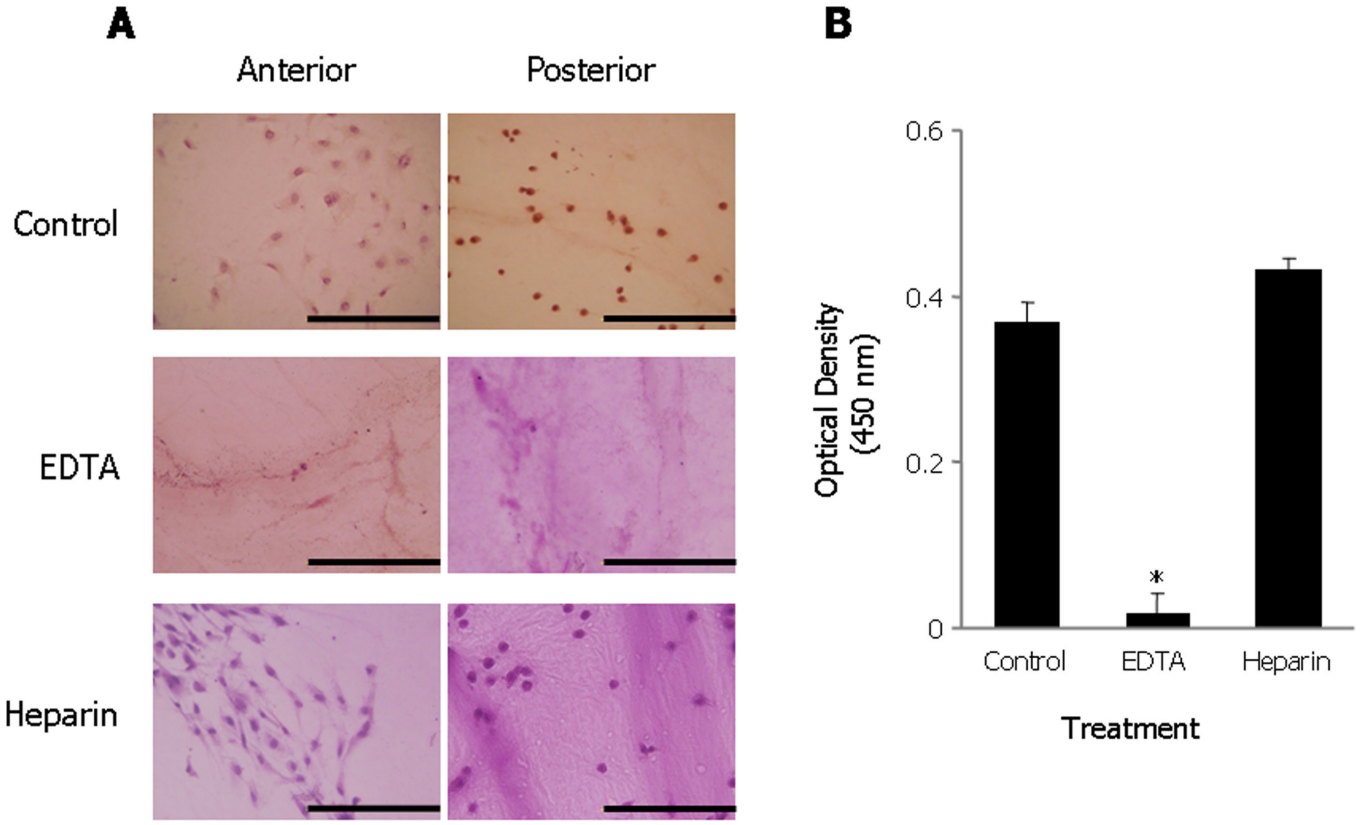

Figure 7. Effect of EDTA and heparin on cell adhesion of endothelial cells. **A:** EDTA (EDTA) or EDTA (5 mM) or heparin (10 µg/ml) was preincubated with bEnd.3 cell suspension for 2 h and then added to the vitreous explants. Thereafter, stereomicroscopic images of bEnd.3 cells on the vitreous surface were taken after hematoxylin and eosin (H&E) staining. The micrograph depicts cell morphology on the vitreous surface in the magnified image. Scale bar=200 µm. **B**: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the number of adherent cells on the vitreous surface. Data are expressed as mean±standard deviation (SD; n=4). *, significantly different from the cell-treated group ($p<0.05$).

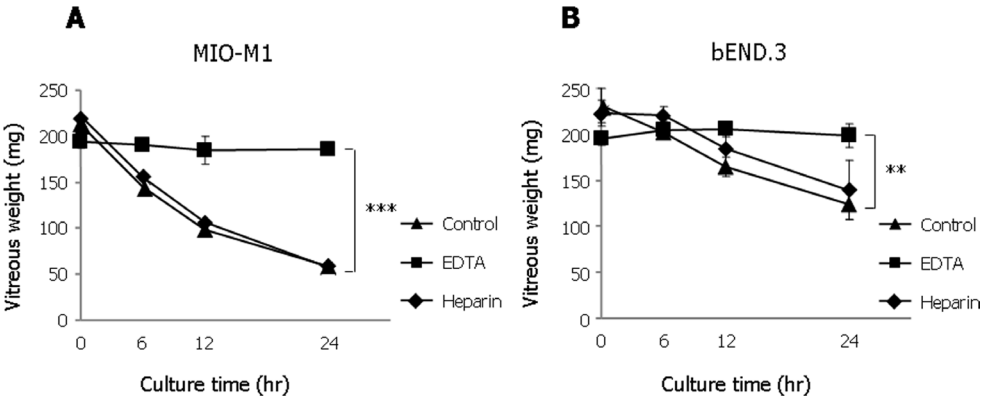

Figure 8. Integrin-dependent contraction of vitreous explants from chicken embryos. EDTA (EDTA; 5 mM) or heparin (10 µg/ml) was preincubated with human retinal Müller cell line (MIO-M1) cell suspension (**A**) and bEnd.3 cell suspension (**B**) for 30 min and then added to the vitreous explants. The vitreous explants were incubated in Dulbecco's modified Eagle's medium (DMEM) at 37 °C up to the indicated periods. Thereafter, the wet weight of vitreous explants was measured for quantitative analysis of vitreous contraction. Data are expressed as mean±standard deviation (SD; n=4). ** and ***, significantly different from control ($p<0.01$ and 0.001, respectively).

related to induction of tractional force on vitreous explants from chicken embryos.

In conclusion, the progression of PVDs cannot be effectively prevented, and the treatment options are limited to vitreoretinal surgery. In addition, postoperative complications such as cicatricial contraction limit the therapeutic success. However, recently, several reports on a candidate compound with pleiotropic function on hyalocytes or retinal pigment epithelial cells, including antiadhesion as well as anticontraction in vitro, have been published [17,53,54]. The application of these candidate compounds to our model is truly interesting. Although this preliminary study must be validated with human vitreous and human RPE cells, we hope that our system will enable the development of an effective pharmacological treatment, which will replace surgical intervention in the near future.

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