• Basic Research •

Regulation of opticin on bioactivity of retinal vascular endothelial cells cultured in collagen

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Abstract

● **AIM:** To investigate the effects of collagen and opticin on the bioactivity of human retinal vascular endothelial cells (hRVECs), and explore its regulations by integrins and RhoA/ROCK1 signal pathway.

● **METHODS:** hRVECs were cultured in collagen and treated by opticin, and cell-based bioactivity assays of cell proliferation, migration, and adhesion were performed. The expression of integrin α2, integrin β1, RhoA and ROCK1 were examined with real-time PCR and Western blotting.

● **RESULTS:** Collagen could promote cell viability of proliferation and migration (all $P<0.05$), and enhance the mRNA expression of integrin α2, integrin β1, RhoA and ROCK1 (all $P<0.05$). Opticin could inhibit proliferation and migration ability of hRVECs cultured in collagen, and reduce the mRNA expression of integrin α2, integrin β1, RhoA and ROCK1 (all $P<0.05$).

● **CONCLUSION:** Collagen and opticin can affect bioactivity of hRVECs, which may be regulated by α2-, β1-integrins and RhoA/ROCK1 signal pathway.

● **KEYWORDS:** opticin; collagen; retinal vascular endothelial cell; integrin; RhoA/ROCK1

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INTRODUCTION

Preretinal neovascularization (PRNV) is an angiogenic process by which new vessels grow from preexisting retinal vasculature into vitreous in conditions including proliferative diabetic retinopathy and retinopathy of prematurity[1]. The new vessels break through retinal inner limiting membrane and then grow in and along cortical vitreous gel, which results in additional pathologic change due to haemorrhage and scar tissue contraction[1]. In previous studies, the collagen on vitreo-retinal interface might play a supporting role in proliferation and migration of neovascularization[2-4]. The posterior vitreous detachment (collapse of vitreous gel away from inner surface of retina) or surgical vitrectomy prevent the new blood vessels from growing in vitreous and sometimes abortive neovascular outgrowths can be seen on inner surface of retina[1]. It is reported that opticin, a small leucine rich repeat protein, bound at surface of vitreous collagens, might influence developmental changes in blood vessels in vitreous cavity and retina, and pathologic processes in which blood vessels grow into vitreous[5-6]. The glycoprotein opticin is an antiangiogenic factor, which inhibits angiogenesis by weakening endothelial cell adhesion to surrounding extracellular matrix[7]. However, its specific signaling pathways and regulatory mechanism of anti-angiogenes is still unsure. In previous study, integrin, a receptor on the surface of retinal vascular endothelial cells, might be the target of collagen-cell binding[8-9]. Integrins are adhesion receptors which transmit signals bidirectionally across plasma membrane[10].

Rho family GTPase-RhoA and its downstream effector Rho-associated coiled-coil containing protein kinase 1 (ROCK1) regulate a lot of cellular processes, including cell adherence, proliferation, permeability and apoptosis[11]. The RhoA/ROCK1 may be an important regulation signaling pathway for endothelial cell-collagen contact, and it may play an essential role in endothelial cell differentiation, network formation and migration[12-13]. Thus, further experimental research should be needed to confirm its specific mechanism.

We hypothesized that collagen may act through interactions with retinal vascular endothelial cells leading to an initiation of proliferation, adhesion, and migration of vascular endothelial cells. Opticin, as an extracellular matrix protein, might influence the bioactivity of vascular endothelial cells in collagen, and the integrins and RhoA/ROCK might be the major signal transmission pathway mechanism. We explored these hypotheses using collagen-based co-culture model in the following study.

MATERIALS AND METHODS

Materials Human retinal vascular endothelial cells (hRVEC; BeNa Culture Collection, China) were used in this study. Materials were obtained from commercial suppliers, as follows: recombinant human opticin protein (Novus Biologicals, USA), cell counting kit-8 (CCK8; Dojindo, Japan), PrimeScript™ RT reagent Kit (Prefect Real Time, TaKaRa Clontech), SYBR® PremixEx TaqTM (Perfect Real Time, TaKaRa Clontech), Modified BCA Protein Assay Kit (Sangon Biotech, China). Collagen type I was obtained from Advanced BioMatrix PureCol. An anti-integrin α2 antibody was from Abcam (USA). An anti-integrin β1/CD29 antibody was purchased from Novus Biologicals (USA). A monoclonal anti-RhoA antibody, a monoclonal anti-ROCK1 antibody and a fluorescently labeled secondary antibodies named m-IgGκ BP-HRP were purchased from Santa Cruz Biotechnology, Inc. (USA).

Anti-integrin α2 antibody (Abcam, USA, ab10800), anti-integrin β1/CD29 antibody (Novus Biologicals, Inc, USA), RhoA (Santa Cruz Biotechnology, Inc, USA), ROCK1 (Santa Cruz Biotechnology, Inc, USA).

Three-Dimensional Collagen Gels Collagen gels were prepared according to Maritan *et al*[14]. Collagen gels were formed by mixing together ice-cold gelation solution [0℃, 10×phosphate buffer saline (PBS), H_2O, type I collagen, 0.1 mol/L NaOH] and adjusted pH of mixture to 7.0-7.5 using sterile 0.1 mol/L NaOH and confirmed with pH paper. Three-dimensional collagen gels were made at a final concentration of 1 mg/mL type I collagen. The gels were allowed to form by incubation in an incubator at 37℃ for 12h.

hRVECs Activities in Collagen hRVECs were purchased from BeNa Culture Collection and cultured in 10% fetal bovine serum-Dulbecco's modified eagle medium (FBS-DMEM; Gbico, USA), respectively according to manufacturer's instructions. They were used between passages 6-10. All cells were incubated in a humidified incubator at 37℃ with 5% CO_2. hRVECs (10^5 cells/mL) were seeded on gel in a volume of 2.5 mL DMEM medium.

Cell Proliferation Assay The number of cells was measured by hemocytometer in collagen-cultured group. Cells were digested with trypsin and collagenase, diluted with trypan blue. We counted the live, unstained cells in one set of 16 squares. All 4 sets of 16 corners were counted. Then, we took average cell count.

Cell proliferation in collagen group and non-collagen group was evaluated by CCK8 assay according to manufacturer's instructions. Cells were cultured 5.0×10^4 cells per 96-well. CCK8 solution of 10 μL was added to each well. Then continue to be incubated for 3h. The absorbance at 450 nm was measured with a microplate reader. Five replicate wells were set.

Cell Migration Assay

Cell spheroids evaluation According to Heiss *et al*[15], the cell spheroid model was established and seeded on surface of collagen gel. Briefly, in a high-glucose DMEM medium containing 0.25% methylcellulose (W/V) (containing 10% FBS and 1% double antibiotics), number of cells was adjusted to 5000 cells/100 μL. Then the medium was inoculated into nonadherent 96-well plate. After 48h, all suspended cells formed a single spheroid per well of defined size and cell number. The spheroids were collected and used for next experiments. Spheroids were generated overnight, after which they were embedded into collagen gel. Then the spheroids which were just in middle of wells were selected for experimental observation. The morphology of cells was photographed under an inverted phase contrast microscope and recorded every 24h for three times. The area (measured by computer software Image pro plus 7) and thickness (measured by gray value in Adobe Photoshop CC software) of cell spheroids were chosen to evaluate the cell ability of migration.

Cell cluster evaluation Cell clustering research model[16] was also used to assess the migration of hRVECs. hRVECs (1×10^5 cells/mL) were distributed evenly on surface of collagen gel, incubated for 24h, recorded every 24h for three times using an inverted phase contrast microscope, and kept track of the same field-of-view. The level of cell migration was shown by the capability of cell distribution from separate to aggregate. In each well, suspended cells were scattered on surface of collagen, and the changes in migration ability were observed by comparing the area of cell clusters measured by computer software Image pro plus 7.

Expression of α2-, β1-integrins, RhoA and ROCK1

Real-time polymerase chain reaction Real-time polymerase chain reaction (PCR) primer sequences used were as follows: integrin α2, 5'-AGACGTGCTCTTGGTAGGTG-3' (forward) and 5'-GCTGACCCAAAATGCCCTCT-3' (reverse); integrin β1, 5'-CGCGCGGAAAAGATGAATT-3' (forward) and 5'-CACAATTTGGCCCTGCTTGT-3' (reverse); RhoA, 5'-CCAGAGGTGTATGTGCCCAC-3' (forward) and 5'-ACAAAGCCAACTCTACCTGCT-3' (reverse); ROCK1, 5'-GGTGGTCGGTTGGGGTATTTT-3' (forward) and 5'-CTCACTTCCCTGTCAGTAAGGA-3' (reverse); GAPDH, 5'-ACGGATTTGGTCGTATTGGGCG-3' (forward) and 5'-CTCCTGGAAGATGGTGATGG-3' (reverse).

Western blotting analysis Western blotting was performed as described previously[17]. Briefly, protein extracts were isolated from each group of cells using RIPA buffer. BCA assay was utilized to measure protein concentration. The following antibodies were used: anti-integrin α2 antibody (abcam, USA, ab10800), anti-integrin β1/CD29 antibody (Novus, USA, NBP2-36561SS), RhoA (Santa Cruz, USA,

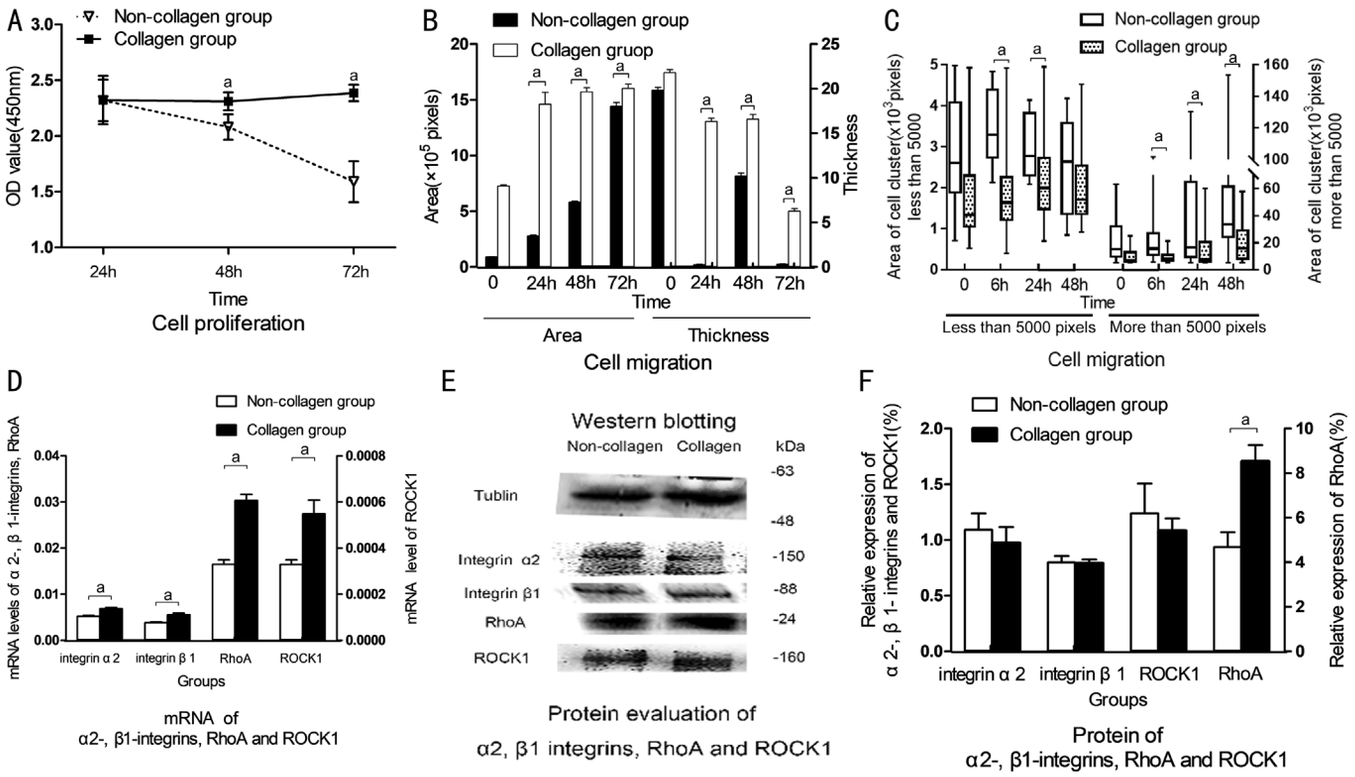

Figure 1 Effects of collagen on hRVECs compared between collagen group and non-collagen group A: Cell proliferation was tested by CCK-8 assay, and 450 nm OD value was evaluated. B: Cell migration evaluated by cell spheroid method. Value was showed as area and thickness of cell spheroids. C: Cell migration evaluated by cell cluster method. The value was showed as areas of cell clusters. D: mRNA levels of integrin α2, integrin β1, RhoA and ROCK1 were tested at 24h after culture. E: Protein expression of integrin α2, integrin β1, RhoA and ROCK1 at 24h after culture were analyzed by Western blotting. F: Values of Western blotting. [a]P<0.05.

sc-418), ROCK1 (Santa Cruz, USA, sc-17794), m-IgGκ BP-HRP (Santa, USA, sc-516102). Total proteins were separated by 8% or 12% SDS-PAGE, and transferred onto nitrocellulose membrane. The membrane was blocked with 5% non-fat milk and subsequently probed with primary antibodies and HRP-conjugated secondary antibodies.

The Effects of Opticin on hRVEC in Collagen When hRVECs were cultured in collagen, opticin was adjusted to a final concentration of 250 nmol/L and added to the medium in opticin group, as well as no opticin added to the medium in non-opticin group. Opticin (250 nmol/L) and anti-integrin α2 antibody (10 μg/mL) were added to the medium in opticin+anti-α2 group, and opticin (250 nmol/L) and anti-integrin β1 antibody (10 μg/mL) were added to the medium in opticin+anti-β1 group. The cell proliferation and migration and cell expressions of integrin α2, integrin β1, RhoA and ROCK1 were detected by above mentioned procedures. Additional assay of cell adhesion ability was taken after the following steps: The cell suspension was inoculated on surface of collagen gel in a 96-well plate (5.0×10^4 cells per well). Wash twice with PBS to wash away the non-adherent cells. CCK8 of 10 μL was added to each well in the dark. While 10 μL CCK8 solution was directly added into control group without solution discarded. After incubating for 3h in the dark, optical density (OD) values were measured at a wavelength of 450 nm by a microplate reader.

Statistical Analysis All experiments were repeated at least three times, and results were expressed as mean±standard deviation (SD) or median (interquartile range) according to characteristics of data. The data were processed by SPSS 25.0. The results of two groups were compared by t-test or Mann-Whitney U test of two independent samples. One-way ANOVA or Kruskal-Wallis H test were used for intergroup comparison. The pairwise comparison between groups was performed using Dunnett's test and SNK test. The test level was α=0.05 with P<0.05 being considered significant.

RESULTS

Effects of Collagen on hRVECs To analyze hRVEC proliferation in collagen, CCK-8 assay was performed. The OD value of CCK-8 assay represented proliferation of hRVEC was increased more significantly in collagen group than in non-collagen group at 48 and 72h after culture (t-test, both P<0.05; Figure 1A).

The area of cell spheroids increased, and the thickness of cell spheroids decreased as time passed. The area and thickness (gray value) of cell spheroids changed significantly in collagen group at 24, 48, and 72h after culture than in non-collagen group (t-test, all P<0.05; Figures 1B, 2A).

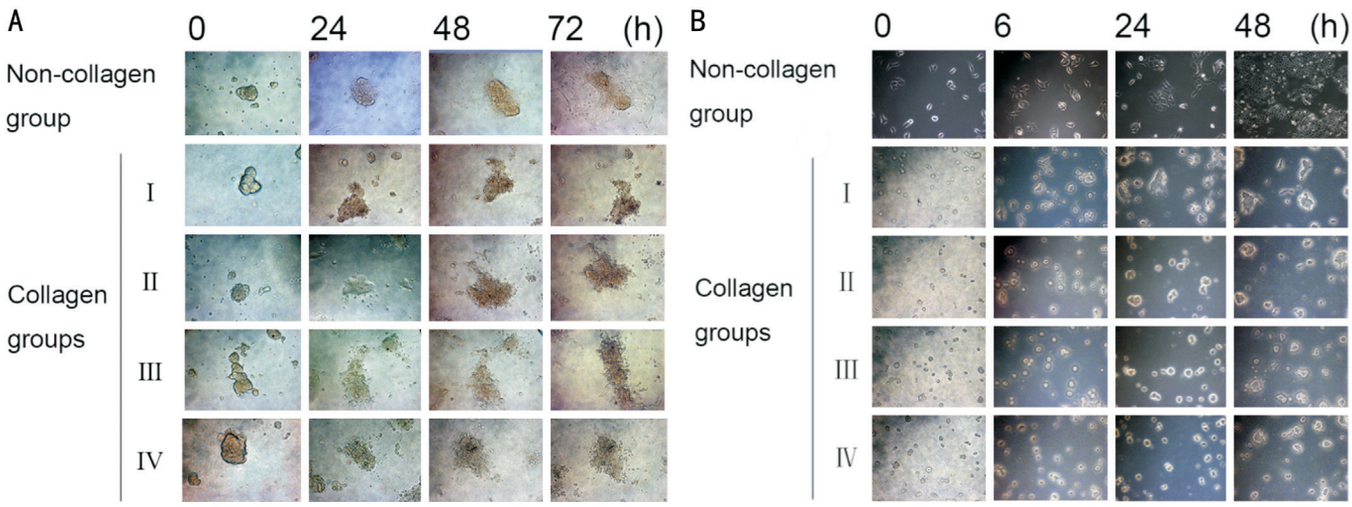

Figure 2 Cell migration evaluation by cell spheroid method (A) and cell cluster method (B) I: Non-opticin group; II: Opticin group; III: Opticin+anti-α2 group; IV: Opticin+anti-β1 group. The area of cell spheroid increased, and the thickness of cell spheroid decreased with time passing (A). The area of cell clusters increased with time passing. Magnification: 200×.

It is a trend of cell cluster for distributed cells. The area of cell cluster should change as time passed. hRVECs were monitored by live cell imaging using inverted phase contrast microscope (Figure 2B). The dimensions of every field of view under microscope are: height, 17.61 cm; width, 23.44 cm; resolution, 150 pixels/inch. We defined the area of one cell cluster more than 5000 pixels means a severe tendency of cell accumulation of hRVECs. The collagen group showed significantly trend to form cell cluster than the non-collagen group at 6, 24, and 48h after culture (Mann-Whitney *U* test, all $P<0.05$; Figure 1C).

Effects of Collagen on α2- and β1-integrins, RhoA and ROCK1 By RT-PCR examination, the mRNA expression of integrin α2, integrin β1, RhoA and ROCK1 were increased significantly in collagen group than in non-collagen group at 24h after culture (*t*-test, all $P<0.05$; Figure 1D). By Western blotting analysis, only the protein expression level for RhoA increased significantly in collagen group than in non-collagen group at 24h after culture (*t*-test, $P<0.05$; Figure 1E, 1F). There was no significant difference in protein expression level for integrin α2, integrin β1 and ROCK1 between collagen group and non-collagen group (*t*-test, all $P>0.05$; Figure 1E, 1F).

Effects of Opticin on Collagen-cultured hRVECs Cell numbers decreased significantly in opticin group, opticin+anti-α2 group and opticin+anti-β1 group after compared with non-opticin group at 24h after culture. Cell numbers decreased significantly in opticin+anti-α2 group and opticin+anti-β1 group after compared with opticin group at 24h after culture (one-way ANOVA, $F=130.055$, $P=0.000$, Dunnett's test, all $P<0.01$). There was no difference in cell numbers between opticin+anti-α2 group and opticin+anti-β1 group (SNK test, $P=0.261$; Figure 3A). The OD value in CCK-8 assay of opticin+anti-α2 group and opticin+anti-β1 group were decreased significantly after compared with non-opticin group or with opticin group at 48h after culture (one-way ANOVA, $F=7.621$, $P=0.002$, Dunnett's test, all $P<0.05$) and 72h after culture (one-way ANOVA, $F=25.36$, $P=0.000$, Dunnett's test, all $P<0.05$) respectively. There was no significant difference among the four groups at 24h after culture (Figure 3B). There was no difference among these four groups in cell adhesion evaluation at 24, 48, or 72h after culture (one-way ANOVA, $F=1.203$, all $P>0.05$; Figure 3C).

Compared with non-opticin group, the area of cell spheroids increased significantly in opticin group, opticin+anti-β1 group, and opticin+anti-α2 group at 24h after culture (one-way ANOVA, $F=205.8$, $P=0.00$), 48h after culture (one-way ANOVA, $F=4234$, $P=0.00$), and 72h after culture (one-way ANOVA, $F=3187$, $P=0.00$, Dunnett's test, all $P<0.05$). Besides, after compared with non-opticin group, the thickness (gray value) of cell spheroids decreased significantly in opticin group, opticin+anti-β1 group, and opticin+anti-α2 group at 24h after culture (one-way ANOVA, $F=4128$, $P=0.00$), 48h after culture (one-way ANOVA, $F=11201$, $P=0.00$), and 72h after culture (one-way ANOVA, $F=499.5$, $P=0.00$, Dunnett's test, all $P<0.05$). There was significant difference in area and thickness of cell spheroids among the four groups at 24, 48, and 72h after culture, except for area comparison between opticin+anti-β1 group, and opticin+anti-α2 group at 72h after culture (Figure 3D).

When the area of cell cluster was less than 5000 pixels, the area of cell clusters increased significantly in opticin group, opticin+anti-α2 group and opticin+anti-β1 group after compared with non-opticin group, as well as in opticin+anti-α2 group and opticin+anti-β1 group after compared with opticin group at 48h after culture (Kruskal-Wallis test, $H=10.55$, $P=0.014$). When area of cell cluster was more than 5000 pixels, the area of cell clusters in opticin group, opticin+anti-α2 group

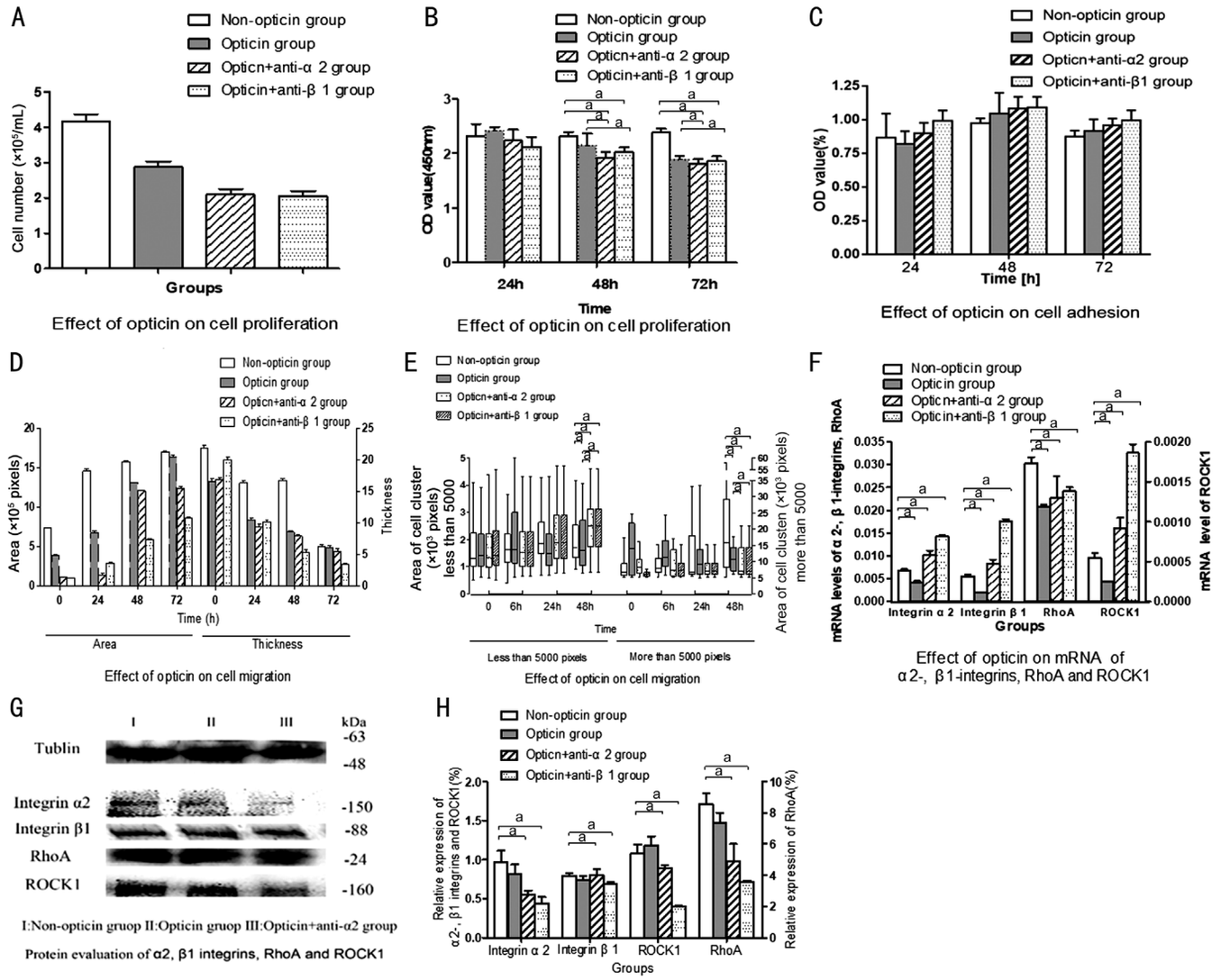

Figure 3 Effects of opticin on collagen-cultured hRVECs compared among non-opticin group, opticin group, opticin+anti-α2 group and opticin+anti-β1 group A: Cell count for cell proliferation evaluation 24h after culture. B: CCK-8 assay for cell proliferation evaluation at 24, 48, and 72h after culture. C: CCK-8 assay for cell adhesion at 24, 48 and 72h after culture. D: Cell migration evaluated by cell spheroid method. Value was showed as area and thickness of cell spheroids. E: Cell migration evaluated by cell cluster method. Value was showed as areas of cell clusters. F: mRNA levels of integrin α2, integrin β1, RhoA and ROCK1 at 24h after culture. G: Protein expression of integrin α2, integrin β1, RhoA and ROCK1 at 24h after culture by Western blotting. H: Values of Western blotting. [a]P<0.05.

and opticin+anti-β1 group, were less than in non-opticin group (Kruskal-Wallis test, H=19.36, P=0.00), as well as the area of cell clusters in opticin+anti-α2 group and opticin+anti-β1 group were less than opticin group (Kruskal-Wallis test, H=10.20, P=0.017) at 48h after culture (Figure 3E).

Effects of Opticin on α2- and β1-integrins, RhoA and ROCK1 The mRNA expression of integrin α2, integrin β1, RhoA and ROCK1 decreased significantly in opticin group, opticin+anti-α2 group and opticin+anti-β1 group than in non-opticin group at 24h after culture (Dunnett's test, all P=0.00; Figure 3F). Besides, the protein expression of integrin α2, integrin β1, RhoA and ROCK1 decreased significantly in opticin+anti-α2 group and opticin+anti-β1 group than in non-opticin group (Dunnett's test, all P=0.00). However, there were no significant differences in the protein expression of integrin α2, integrin β1, RhoA and ROCK1 in non-opticin group, opticin+anti-α2 group and opticin+anti-β1 group, after compared with opticin group (SNK test, all P>0.05; Figure 3G, 3H).

DISCUSSION

Previous studies stated that extracellular matrix (ECM) is surprisingly versatile and dynamic and can profoundly affect behaviors of cells such as vascular endothelial cells[18]. ECM might play a central role in angiogenesis and certain ECM components including collagen, fibronectin or fibrin are required for vascular endothelial cell migration and tube morphogenesis[19]. As new vessels form, they lay down a basement membrane that surrounds the endothelial tubes and is essential for their stability[1]. Collagen, a major component of vitreous gel, being the main natural ECM component, may be

an ideal choice for an engineered scaffold material, as it has the advantage of providing both structural and microenvironmental support, the latter through storage and delivery of biologically active factors[20]. Thus, vascular endothelial cell could pull on the substratum at focal adhesions, which generates forces that are required for locomotion, and cell ability of proliferation or migration could be promoted[21]. In this study, the intraocular gel condition of vitreous was mimicked by a collagen-based culture model *in vitro*, and the activities of hRVEC including cell proliferation, migration and adhesion which are critical process for angiogenesis were assessed[22]. Here we found, proliferation ability of hRVEC were promoted more significantly in collagen-cultured group than non-collagen group, which was consistent with other research[20,23-24]. As to assessment of cell migration ability, the most commonly used and earliest developed methods *in vitro* is wound-healing assay, which need creating a "wound" in a cell monolayer (etching lines lightly with a razorblade on the bottom of dish)[25]. But the assessment method of wound-healing assay is not suitable to be adopted for three-dimensional collagen matrices culture model in this study, and methods of cell spheroids evaluation and cell clustering research model were chosen to assess cell migration ability. It was evaluated by the change of cell spheroid areas, because cells could migrate out of accumulated-cell spheroids resulted in radial extension of cell-covered area[15], which was firstly reported by Korff and Augustin[26]. Some studies also successfully used high-cell-density tissue spheres and quantified spheroidal sprouting of cell spheroids to observe cell migration ability[27-29]. Besides, cell clustering and migration research model for cells cultured on surfaces of three-dimensional collagen matrices were firstly reported by da Rocha-Azevedo and Grinnell[30]. After initial cells attachment to collagen matrix, cells could migrate closer to each other with small cell clusters. Here we found, the migration ability of hRVEC, assessed by both cell spheroids evaluation and cell clustering model, were promoted more significantly in collagen-cultured group than non-collagen group, which was consistent with other research[31]. However, it is difficult to compare cell adhesion ability of hRVEC between collagen-cultured group and non-collagen group because of their different culture surfaces adhered by cells. Cell adhered to plastic surfaces of wells directly in non-collagen group, and to the collagen-coated surfaces of wells in collagen group. Here, we found that among collagen-cultured groups, cell adhesion ability of hRVEC were not changed significantly by effect of opticin or antibody against integrins.

In order to reveal the regulation of angiogenesis induced by collagen, we explored the effects of opticin in this study. Opticin is a member of the small leucine rich repeat proteoglycan family and is localized particularly in certain extracellular matrices such as around the vitreous collagens. It binds collagen fibers non-covalently to maintain collagen structure stability, and its fluorescently labeled antibody even could be detected on the surface of vitreous gels[32]. Besides, opticin possesses a special intraocular anti-angiogenesis character, which could be even balanced by vascular endothelial growth factor-A. Probably opticin inhibits vitreous collagen interactions with vascular endothelial cell, thereby regulating the proangiogenic signaling *via* collagen. In this study, the cell proliferation and migration ability of hRVEC in opticin-treated group was decreased significantly than in non-opticin group. Therefore, opticin probably could regulate biological activity and function of hRVEC when cultured with collagen. In order to explore signal transmission pathway of collagen-induced and opticin-regulated neovascularization, we hypothesized that integrins and RhoA/ROCK1 take essential roles in it. Integrin is a transmembrane protein composed of α- and β-subunit dimers. It is well known that the α subunit of the lateral end is recognized with ECM or its ligand, while β subunit of the medial end is connected to the cytoskeleton through actin, which induces the expression of neovascularization cell behaviors such as adhesion, migration, division and proliferation along scaffold of the extracellular matrix[33]. Integrin family consists of 24 α-β heterodimer members, some of which mediate attachment of cells to ECM and participate in specific cell-to-cell interactions. Primarily the collagen-binding integrins are known to belong to β1 subfamily[34]. Further study reported that inhibition of integrin α2 will affect the formation of neovascularization of endothelial cells[7]. In previous study, integrin α2β1 was reported to play a major role in signal pathway of collagen-induced neovascularization. Integrin α2β1 has been found to be expressed in vascular endothelial cells[35], and acts as a high affinity binding site between collagen and vascular endothelial cells[34,36-37]. Le Goff *et al*[7] found that collagen stimulates angiogenesis through continuous signal transduction of α2β1 integrin expressed on endothelial cells, leading to initial and key changes in cell activities. Therefore, it is still unclear which one of the two subunits, α2 or β1, or both are the real signal transmission factors. In this study, the levels of integrin α2 and integrin β1 were increased significantly in hRVECs cultured with collagen than in hRVECs cultured without collagen. Among opticin-treated groups, the proliferation and migration ability of hRVECs were inhibited significantly both in anti-integrin α2 group and anti-integrin β1 groups. Besides, in collagen-cultured groups, the expression levels of integrin α2 and β1 were decreased significantly in hRVECs treated with opticin than treated without opticin. Therefore, both the two subunits, α2 and β1, might be the real signal transmission factor for collagen induced angiogenesis. Opticin might inhibit vascular

endothelial cell interactions with collagen *via* integrin α2 and β1, which is consistent with the other research.

RhoA, a small GTPase, is a universal regulator of many cellular processes including cell adhesion, vesicle transport, proliferation, survival, cell morphology, and cell-matrix interactions[12]. ROCK)has been identified as the main downstream effector of RhoA. Recent data showed that RhoA/ROCK1 signaling pathway is involved in various physiological and pathological processes of neovascular diseases, and is an important component of the pathogenesis of preretinal neovascularization diseases such as proliferative diabetic retinopathy[12-13]. In this study, the levels of RhoA and ROCK1 in hRVECs cultured with collagen were increased significantly than cultured without collagen. Among collagen-cultured groups, the levels of RhoA and ROCK1 showed decreased expression in hRVECs treated by opticin than treated without opticin. Among collagen-cultured and opticin-treated hRVECs, the levels of RhoA and ROCK1 were increased significantly both in anti-integrin α2 and anti-integrin β1 groups than in without anti-integrin antibody group.

In this study, to evaluate the effects of collagen on hRVECs, both mRNA and protein expression of integrin α2, integrin β1, RhoA and ROCK1 were analyzed. Collagen could increase the mRNA level of integrin α2, integrin β1, RhoA and ROCK1, but only increased protein expression of RhoA. But the elevation of mRNA level in collagen-cultured hRVECs did not translate into equally highly elevated protein levels. The possible reason might be that mRNAs are not always a direct indication of protein levels. protein might be regulated not only on transcription level, but also regulated post-translationally on its different degraded and re-synthesized rates. Besides, it is possible that all those protein levels were changing, but the changes were too subtle to be detected using Western blotting. However, in the following effect evaluation of opticin on collagen-cultured hRVECs, both mRNA and protein level of integrin α2, integrin β1, RhoA and ROCK1 were elevated simultaneously in opticin group, opticin+anti-α2 group and opticin+anti-β1 group more than in non-opticin group.

Therefore, in this *in vitro* study, the opticin, a structural protein, might be inhibitor of collagen-induced angiogenesis by regulating the interaction between collagen and vascular endothelial cells, and effects of α2- and β1-integrins and RhoA/ROCK1 signaling pathway might be one of its inner mechanisms.

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