·Basic Research·

Inhibition of proliferation of retinal microvascular endothelial cells by pericytes through down-regulating KDR/Flk-1 in a co-culture system

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Abstract

• AIM: To investigate the role of pericytes in growth of retinal microvascular endothelial cells(RMECs) in a co-culture system in order to understand some mechanism of angiogenesis in hypoxia induced retinal neovascular disorders.

• METHODS: RMECs were isolated by a modified protocol using CD_{31} coated Dynabeads, and identified by immunocytochemical staining with anti-Factor VIII and CD_{31} antibodies. Rat retinal pericytes were isolated and characterized by immunofluorescent staining with PDGFR-β ; and desmin antibodies. Pericytes and RMECs were cultured in a contact co-culture system both under normoxia and hypoxia by Millicell chamber. RMECs proliferation was evaluated by MTT and cell cycle assay with flow cytometry. RT-PCR was used to detect the alteration of KDR/Flk-1 mRNA level in RMECs under normoxia or hypoxia in the co-culture system.

• RESULTS: Highly pured rat RMECs and pericytes were harvested with the modified isolating method. The two cell types were identified by positive Factor VIII, CD_{31} and PDGFR-β , desmin cytochemical staining respectively. RMECs proliferated significantly under hypoxia from 3 to 9 day with a maximal rate on day 6 (24.9%, $P < 0.01$) by MTT. In the co-culture system, the proliferation of RMECs was inhibited by pericytes. After 6 days exposure to hypoxia, the fraction of S-phase RMECs number was greatly increased by 43.9%($P < 0.01$). In the co-culture system, RMECs proliferation was inhibited by pericytes through decreasing the fraction of S-phase cell number both under normoxia (3.6%, $P<0.05$) and under hypoxia (15.1%, $P <0.01$). KDR/Flk-1 mRNA level in single cultured RMECs was shown to increase approximately 1.3-fold when exposed to hypoxia. Compared with single cultured RMECs, co-culture with pericytes could decrease KDR/Flk-1 mRNA by 45.1% ($P <0.05$) and 27.7% ($P<0.05$) under normoxia and hypoxia condition respectively.

• CONCLUSION: The present study demonstrated that pericytes could inhibit proliferation of RMECs under both normoxia and hypoxia. The inhibition effects of pericytes maybe, at least in part, due to downregulation of KDR/Flk-1 of RMECs. These findings confirm that pericytes could be a potential inhibitor in the pathogenesis of retinal neovascularization(RNV).

• KEYWORDS: pericytes; retinal microvascular endothelial cells; co-culture; proliferation; KDR/Flk-1; angiogenesis; hypoxia

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INTRODUCTION

Retinal neovascularization (RNV) is a major cause of blindness in widespread retinal diseases such as diabetic retinopathy (DR), retinopathy of the prematurity (ROP) and retinal vein occlusion (RVO). Formation of new vessels in retina is a complex multi-step process including proliferation, migration and differentiation of retinal microvascular endothelial cells (RMECs). Pericytes, the other components of capillaries, intimately embracing capillary endothelia, are suggested to regulate the growth of these cells [1,2]. One of the initial observations leading to this hypothesis was the selective loss of pericytes from the retinal microvasculature, which was prior to the onset of neovascularization in DR [3]. This indication is supported by the fact that the arrival of the pericytes into newly formed capillaries were shown to mark the cessation of vascular growth during wound healing [4]. These *in vivo* observations indicate that pericytes may inhibit proliferation of

endothelial cells. A few *in vitro* studies demonstrated that pericytes could inhibit endothelial cells growth in a contact-dependent manner [5,6]. But endothelial cells in these studies originated from bovine adrenal cortex or cell lines, which are different from retinal endothelial cells for their high organ-specificity and heterogeneity [7]. So far, more investigations are needed to elucidate the effects of pericytes on retinal endothelial cells. Although it is known that hypoxia is a leading cause of RNV, the effect of pericytes on RMECs proliferation under hypoxia still remains unclear to date. Exploring whether pericytes could inhibit RMECs proliferation under hypoxia will provide better understanding of the role of pericytes in RNV. Furthermore, little is known about the mechanism of pericytes' inhibition effect. It is accepted that vascular endothelial growth factor (VEGF) plays an important role in the hypoxia-stimulated neovascularization of ischemic retinal diseases such as proliferative diabetic retinopathy (PDR). VEGF exerts its effect through high-affinity tyrosine kinase receptors, named kinase insert domain-containing receptor (KDR/Flk-1), which located primarily on endothelial cells. Therefore the alteration of KDR/Flk-1 on RMECs was involved in our experiments. The present study aimed at investigating the role of pericytes on endothelial cell proliferation both under normoxia and hypoxia in a co-culture system. Furthermore, the expression of KDR/Flk-1 on RMECs was also evaluated to reveal the possible underlying mechanism.

MATERIALS AND METHODS

Cell Culture and Identification of RMECs and Pericytes

Retinal microvessels were obtained from male Wistar rats, weighing 250-300g (Animal Center of the Fourth Military Medical University). Primary RMECs were isolated from the fresh retinae as previously described [8,9] with affinity purification using magnetic beads coated with anti-CD31 antibody. Mouse anti-rat CD_{31} antibody (Chemicon, Temecula, CA, USA) was incubated with beads pan mouse IgG (Dynal Biotech, Lake Success, NY, USA) overnight at 4℃ to prepare magnetic beads coated with anti-CD_{31} antibody. Retina were removed aseptically from eyes under a dissecting microscope. Retinae were pooled together, then minced and filtered through a 53μm nylon mesh. The filtrate was discarded and the mesh rinsed in Dulbecco's modified Eagle's medium (DMEM, Gibco, US) to suspend the tissue and then centrifuged. The supernatant was discarded and the pellets were suspended and digested in 1g/L collagenase type I in serum-free DMEM for 30 minutes at 37℃ with agitation. The cellular digests were filtered through a 30μm nylon mesh, and then centrifuged. The pellets were suspended in DMEM containing 100mL/L fetal bovine serum (FBS) and incubated with magnetic beads coated with anti-CD_{31} antibody for 30 minutes at 4℃. After affinity binding, magnetic beads were washed six times with DMEM/100mL/L FBS. The cells in eluant were centrifuged and suspended in pericytes growth medium followed by seeding onto culture flask(Costar, USA) [10]. Magnetic beads binding cells in the endothelial cell growth medium were seeded onto rat tail collagen type I-coated tissue culture flask. RMECs grew in DMEM supplemented with 100mg/L FBS,20mmol/L sodium bicarbonate,100kU/L benzylpenicillin sodium and 100kU/L streptomycin sulfate, freshly added heparin at 55kU/L (Sigma, US) and endothelial cell growth supplement 100μg/mL (ECGS) (Sigma, US) (culture medium-A). The culture medium-B for pericytes consisted of culture medium-A without heparin, ECGS and 200mL/L fetal bovine serum instead. Both cells were cultured in a humidified incubator of 50mL/L CO_2/950mL/L air at 37℃ and culture medium was replaced every three days. Cells between passages 2 and 6 were used in this experiment. RMECs were identified by polyclonal rabbit VIII antibody (1:100, Santa Cruz) staining and monoclonal mouse anti-rat CD_{31} antibody (1:100 in PBS-plus, Chemicon) plus Cy3-conjugated second goat anti-mouse antibody. Pericytes were identified by rabbit anti-PDGFR-β; antibody (1:100, Santa Cruz) and mouse anti-rat desmin (1:100, Dako) with FITC or Cy3-conjugated second antibody respectively.

Pericyte and RMEC Co-culture System and Hypoxia Treatment

Co-culture model Millicell insert (0.4μm, Millipore) was used to develop a contact co-culture system. Pericytes were seeded (1×10^7/L) on the backside membrane of a rat tail collagen type I -coated cell culture insert and cultured for 24 hours. After that, the insert was turned over and RMECs were seeded (2×10^7/L) on the upper side of the insert. In the single culture, RMECs were seeded on the upper side of the insert. Experiments with the co-culture model were performed within 3 to 9 days after RMECs were seeded.

$CoCl_2$ induced hypoxia condition Hypoxic conditions were achieved by adding $CoCl_2$ (200μmol/L) to medium to mimic hypoxia [11]. The growth medium was renewed every three days and $CoCl_2$ was added on 3, 6 and 9 day.

Experimental Groups RMECs under varies treatment were divided into four groups as follows: RMECs cultured under normoxia (EC/N), RMECs cultured under hypoxia (EC/H), RMECs and pericytes co-cultured under normxia (Co/N), and RMECs and pericytes co-cultured under hypoxia (Co/H).

Cell Growth Analysis with MTT Assay RMECs or RMECs (2×10^7/L) with pericytes (1×10^7/L) were seeded on the 24-well Millicell chamber. Afer 24 hours culture, the viability of RMECs in single culture or co-culture groups were estimated using 3- [4, 5-dimethylthiazol-2-yl]-2, 5diphenyltetrazolium bromide (MTT) (Sigma, USA), which is based on the reduction of soluble yellow MTT tetrazolium salt to a blue insoluble MTT formazan product by mitochondrial succinic dehydrogenase [12]. The amount of formazan product is proportional to the number of viable cells. MTT was added to each well to a final concentration of 0.5g/L and incubated for an additional 4 hours. Supernatant was removed and formazan crystals were dissolved in 200μL dimethyl sulfoxide, then optical density of the mixture was measured spectrophotometrically at 490nm. Cellular viability was determined as percent of activity in control wells. On day 3, 6 and 9, MTT assay was carried out to determine the number of cells in each well.

Cell Cycle Analysis with Flow Cytometry RMECs cultured on day 6 were washed with 0.01mol/L PBS and harvested with a rubber policeman. Then Cells were washed in PBS containing 10mL/L FBS(10mL/L FBS/PBS), suspended in the same buffer to a concentration of 5×10^6-1×10^7 cells/mL. After that, cells were fixed in 70% ethanol and stored at 4℃ for at least 1hour and up to 14 days before staining with propidium iodide (PI). For PI staining, cells were washed in 10mL/L FBS/ PBS prior to incubation at 37℃ for 30 minutes in the same solution containing 100mg/L PI (Molecular Probes). All samples were tested on a FACScan flow cytometer equipped with 488nm argon laser (Becton Dickinson). Data were collected and analyzed using CellQuest software. A minimum of 5 000 cells were assayed for each sample.

RT-PCR Analysis Total RNA was extracted from RMECs cultured on day 6 using an RNeasy kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Primers for KDR/Flk-1 were: sense 5'- CAGAAAA GGAGATGCCC GAC-3' and antisense 5'- TCCAGAGT TTTCAGCTCTTC -3' with an expected product of 300bp [13]. Primers for GAPDH were: sense 5'-TCCCACC ACCCTGTTGCTGTA-3' and antisense 5'-ACCACAG TCCATGCCATCAC-3', with an expected product of 455bp [14].RT-PCR was carried out using a reaction system (TakaRa one step RNA PCR kit, Japan) composed of sample RNA 1μg, specific primers 1μL, 10×Tris-HCl buffer 5μL, $MgCl_2$ (25mmol/L) 10μL, 10mmol/L dNTP Mixture5μL, 40U/μL RNase inhibitor 1μL, 5U/μL AMV reverse transcriptase 1μL, and 5U/μL AMV-Optimized Taq 1μL in a final volume of 50μL. Reverse transcription was carried out at 94℃ for 30 minutes and inactivation at 50℃ for 2 minutes. Then samples were submitted to PCR amplification as follows: KDR/Flk-1: 30 cycles of denaturation at 94℃ for 30 seconds, annealing at 62℃ for 30 seconds, and extension at 72℃ for 90 seconds. GAPDH: 35 cycles of denaturation at 94℃ for 30 seconds, annealing at 59℃ for 30 seconds, extension at 72℃ for 60 seconds, and ended with elongation at 72℃ for 5 minutes. PCR-amplified products were visualized in a 1.5% agarose gel stained with ethidium bromide. DNA ladder (DL2000 Marker, TakaRa, Japan) was loaded on each gel. The density of individual lanes was normalized to the density of the PCR amplified internal control GAPDH.

Statistical Analysis For all assays, three or more separate experiments were performed. Mean SD from several experiments were calculated. One-way ANOVA was used to assess statistical significance of differences among means of more than two groups. The level of statistical significance was set at a P value of 0.05.

RESULTS

RMEC and Pericyte Co-culture Model In the present experiment, modified protocol using CD_{31} coated Dynabeads was used to isolate primary cultured rat (RMECs) (Figure 1A). RMECs with high purity and activity were successfully harvested by this method. During the following 2-3 weeks, RMECs clone formed and reached confluence for about 4-5 weeks (Figure 1B). Furthermore, RMECs were identified by immunocytochemical staining using anti-Factor VIII antibody and anti-CD_{31} antibody (Figure 1C,D). Rat retinal pericytes were isolated by selective culture condition and could passage continuously. Pericytes showed irregular shapes with thick filament in the cytoplasm and overlapping growth pattern without contact inhibition (Figure 2A). Pericytes were characterized by immunofluorescent staining with PDGFR-β; and desmin antibody (Figure 2B,C and D) detected with laser scanning confocal microscopy. Pericytes

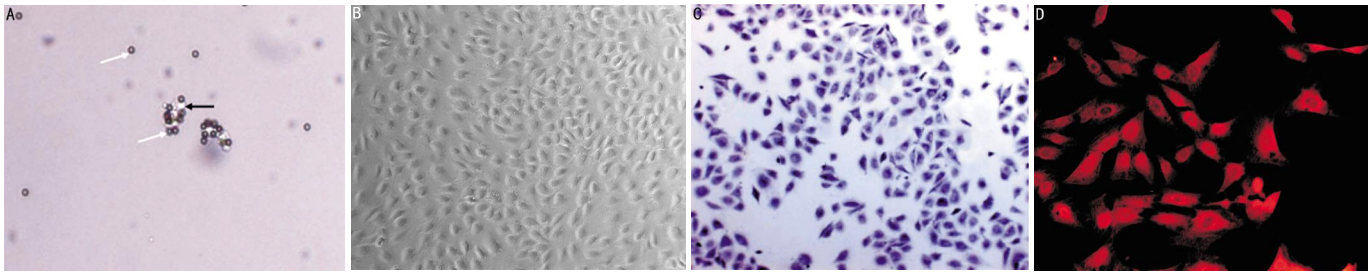

Figure 1 Morphology and identification of rat retinal microvascular endothelial cells(RMECs) A:Dynabeads (white arrow) isolated RMECs (black arrow) clump 24 hours after primary culture with phase-contrast microscopy × 200;B:The appearance of RMECs in a confluent monolayer with phase-contrast microscopy ×100;C:Immunocytochemical staining with anti-vWF antibody DAB ×100;D:Immunofluorescence staining with anti-CD31 anibody Cy3×100

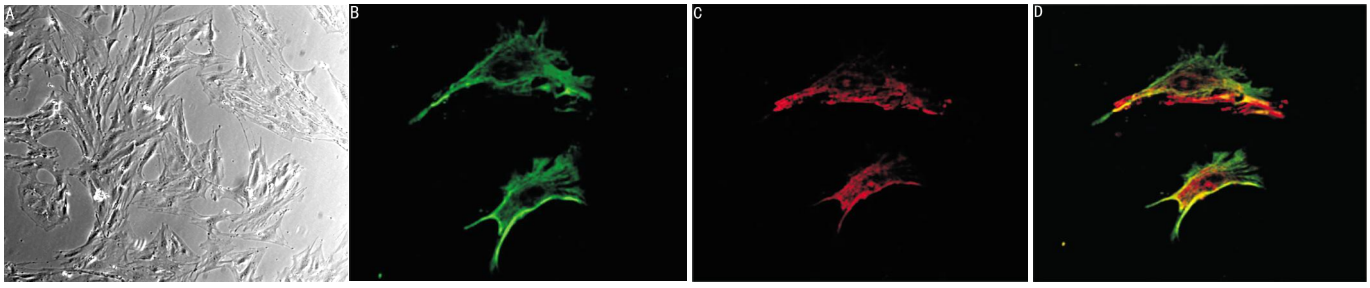

Figure 2 Morphology and identification of rat retinal microvascular pericytes A:Photograph of phase-contrast microscopy of passaged rat pericytes, showing non-contact-inhibited growth with irregular shapes and fiber bundles ×100;B:Positive fluorescence staining of PDGFR-β with laser scanning confocal microscopy ×200;C:Positive fluorescence staining of desmin with laser scanning confocal microscopy×200;D:Positive fluorescence staining of both PDGFR-β and desmin×200

Table 1 Inhibition of rat RMECs growth in each group with MTT assay

Group	3d		6d		9d	
	A(%)	GIR	A	GIR(%)	A	GIR(%)
EC/N	0.173±0.016	-	0.185±0.015	–	0.193±0.019	–
EC/H	0.198±0.020[a]	-14.5	0.231±0.022[b]	-24.9	0.228±0.022[b]	-18.1
Co/N	0.156±0.019[a]	9.8	0.152±0.016[b]	17.8	0.163±0.022[a]	15.5
Co/H	0.181±0.023	-4.6	0.206±0.015[a]	-11.4	0.211±0.018[a]	-9.3

GIR: Growth inhibition ratio=(control – treatment)÷ control ×100%;[a]P<0.05 *vs* EC/N;[b]P<0.01 *vs* EC/N

and RMECs were cultured in a contact co-culture system by Millicell chambers, with RMECs growing on the upper side of the insert membrane and pericytes on the backside. RMECs and 0.4μm pores in the membrane could be seen clearly (Figure 3). These pores allowed the soluble material to pass freely.

Cell Growth Analysis with MTT Assay In order to examine the effects of pericytes on proliferation of RMECs under normoxia or hypoxia, the number of RMECs was analyzed with MTT assay, which was normalized with growth inhibition ratio (GIR) (Table 1). The number of RMECs treated with hypoxia showed a significant increase of about 14.5 % (P<0.05) within 3 days. The cell number reached its peak on day 6 after exposure to hypoxia (24.9%,

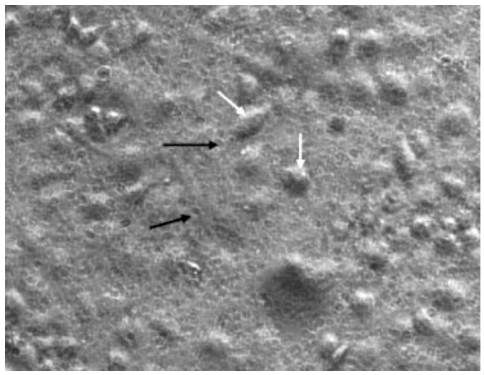

Figure 3 The co-culture system of rat retinal microvascular endothelial cells (RMECs) and pericytes. RMECs (white arrow) were attached and flatted to the upper side of Millicell insert membrane, with many 0.4μm pores (black arrow) in the membrane under a phase-contrast microscope ×200

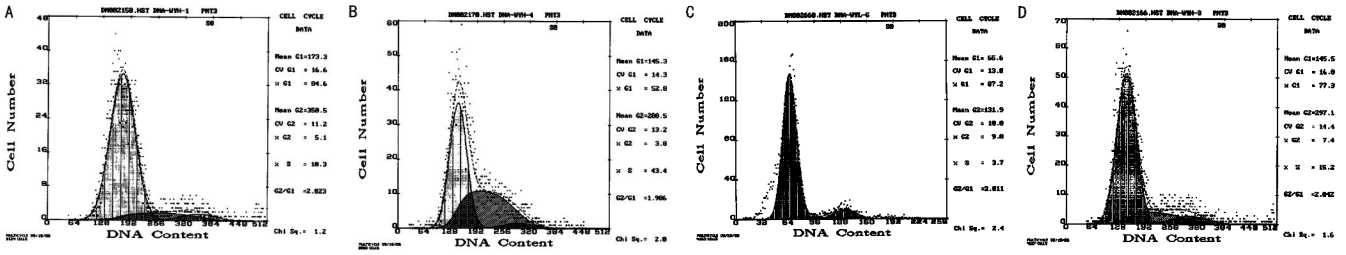

Figure 4 DNA cycle assay of retinal microvascular endothelial cells in each group with flow cytometry

P<0.01) and declined on day 9. Negative value of GIR indicated that hypoxia induced proliferation of RMECs. In the co-culture system under normoxia, proliferation of RMECs was significantly inhibited by about 9.8%, 17.8% and 15.5% on day 3, 6 and 9 respectively and followed the same trend as described above. Hypoxia could also significantly inhibit RMECs' proliferation in the co-culture system on day 3, 6, and 9. GIR values were still negative in the co-culture system under hypoxia.

Cell Cycle Analysis with Flow Cytometry On day 6, cell cycle analysis using FCM was applied to further investigate the proliferation of RMECs (Figure 4, Table 2) as indicated by MTT. It was observed that hypoxia could significantly increase the fraction of S-phase RMECs number by 43.9% (P <0.01). Under normoxia, pericytes inhibited RMECs proliferation through decreasing the fraction of S-phase cell number by about 3.6%(P <0.05).When exposed to hypoxia, pericytes in the co-culture system inhibited the increasing of S-phase fraction prominently (from 43.9% to 15.1%,P<0.01). However, the inhibition was not complete as S-phase cells in Co/H were still a bit more than in EC/N group.

KDR/Flk-1 mRNA Levels in RMECs of Each Group The influence of pericytes on KDR/Flk-1 expression in RMECs under normoxia or hypoxia was further investigated at mRNA level by RT-PCR (Figure 5)KDR/Flk-1 mRNA in single cultured RMECs reached evidently at an approximately 2.3-fold when exposed to hypoxia. KDR/Flk-1 mRNA in co-cultured RMECs significantly decreased by 45.1% and 27.7% under normoxia and hypoxia condition respectively, compared with single cultured RMECs (Figure 6). It suggested pericytes could decrease KDR/Flk-1 mRNA level under normoxia and partly inhibit hypoxia induced KDR/Flk-1 mRNA expression of RMECs.

Table 2 Cell cycle assay of retinal microvascular endothelial cells in each group

Group	G_1(%)	S(%)	G_2(%)	G_2/G_1
EC/N	84.1 ± 2.8	10.5 ± 0.6	5.5 ± 0.4	2.023 ± 0.02
EC/H	52.1 ± 3.5	43.9 ± 0.8[b]	3.8 ± 0.3	1.986 ± 0.01
Co/N	88.3 ± 4.1	3.6 ± 0.1[a]	9.1 ± 0.6	2.011 ± 0.02
Co/H	77.9 ± 3.2	15.1 ± 0.9[a]	7.6 ± 0.3	2.042 ± 0.02

[a]P<0.05 *vs* EC/N,[b]P<0.01*vs* EC/N

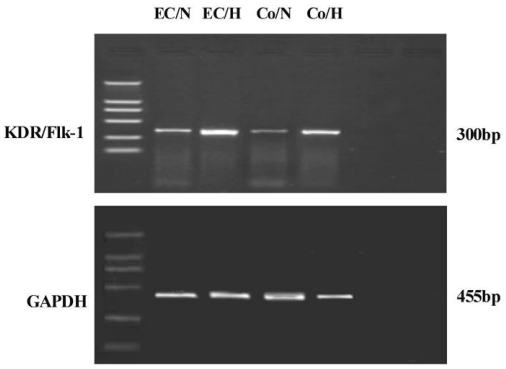

Figure 5 KDR/Flk-1 mRNA product of RMECs in various group, with GAPDH control

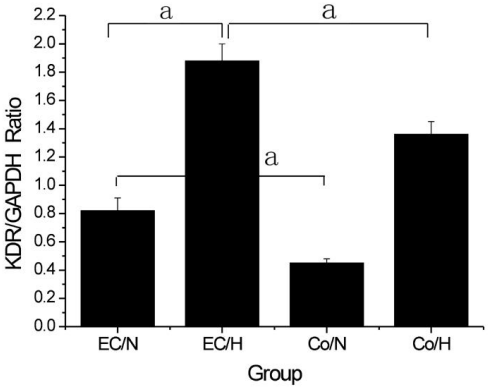

Figure 6 Quantification KDR/Flk-1 mRNA levels in each group by densitometry. KDR/Flk-1 mRNA expression was normalized to GAPDH. Data were means SD from n=3 experiments (P< 0.05)

DISCUSSION

Morphological studies of developing capillaries and observations of pathologic neovascularization indicate that pericytes may act as suppressors of endothelial cells growth. To detect this potential effect of pericytes, *in vitro* pericyte-EC interaction strategies were adopted. Martin *et al* [15] suggested that pericytes conditioned medium could

significantly inhibit proliferation of human microvascular endothelial cell line by releasing soluble inhibitors. By contrast, Kondo *et al* [5] demonstrated that TR-rPCT1 cells (immortalized retinal pericytes line) could only inhibit proliferation of TR-iBRB2 cells (immortalized retinal endothelial cell line) by contact co-culture system and have no effect on non-contact co-culture system. Orlidge *et al*[6] also suggested that pericytes could exert their inhibition effect only through direct contact. The model they used was putting the two types of cells growing in the same culture plate. Limits of these models were therefore apparent as that pericytes conditioned medium was only viable to study the transient effect of soluble factors and lost important information regarding pericyte-EC contact. What's more, direct contact achieved through one-plate co-culture was far different from capillary structure and the growth of both cells was difficult to control. As is well known, pericytes are embedded in basement membrane and stretch out processes through base membrane to contact with underlying endothelial cells instead of direct cell contact. Millicell chamber is an ideal facsimile of this condition as it prevents direct contact but allows processes or soluble factors to go through its pores. This co-culture model, established between rat microvascular endothelial cells and rat retinal pericytes，provides us more information and easier growth control for both kinds of cells. It was observed that pericytes could always inhibit ECs' growth with varying pericyte/EC ratio (from 1:1 to 1:10), which may reflect that pericytes coverage in EC surface varies extensively in different tissues [16]. The highest coverage ratio has been described in the retina (50%), which prompts us to take the 1:2 pericyte/EC ratios in this study. And it worked by showing the inhibition effect as well as not inhibiting RMECs growth destructively. Our study confirmed that pericytes could significantly decrease RMECs number as well as their DNA synthesis throughout the 9-day time course in the co-culture model. Ultrastructural studies had demonstrated that pericytes formed at least three types of junctions with ECs [17]. Using dye transfer as an indicator of gap junctions, Larson *et al*[18] showed that junctional communication between microvascular pericytes and ECs occurred in vitro. Those findings indicated that pericytes might exert inhibition effect on RMECs in our co-culture system through forming gap junctions with RMECs. Extracellular matrix may also play a role in the inhibition of EC growth. Basement membrane components were shown to modulate the growth, migration, and differentiation of ECs [19-21]. Pericytes could synthesize basement membrane components, such as fibronectin, IV collagen and multiple laminin isoforms [22,23]. Morphologic studies of developing capillaries correlate the arrival of pericytes with the cessation of EC migration and proliferation, a shift in dominant populations of matrix components [24,25]. Thus, influence on capillary basement membrane posed by pericytes may be one mechanism for modulation of EC proliferation. Angiogenesis occurs not only during the process of physiological conditions but also in various pathological conditions known as pathological neovascularization. Hypoxia is the principal factor inducing RNV via ECs proliferation, migration, differentiation [26-28]. Till now, there are only several indications that pericytes deficiency led to reduced inhibition of endothelial proliferation in hypoxia induced ROP model *in vivo* [29]. Up to date, little direct evidence is obtained about the role of pericytes on proliferation of endothelial cell in retina under hypoxia, which is important for clarifying the pathogenesis, prevention and treatment of RNV. Our results determined that pericytes could decrease RMECs numbers as well as inhibit their synthesis of DNA under hypoxia. Although the inhibition posed by pericytes was not completely and declined after 6 days co-culture. Cell contact and/or molecules secreted by pericytes may play a role in this process. VEGF is a pivotal stimulatory factor during retinal vascularization and the activity of VEGF is mediated by specificity binding to its receptors. VEGF isoforms bind two types transmembrane protein-tyrosine kinase receptors, Flt-1 (fms-like tyrosine kinase)[30] and KDR/Flk-1 (kinase domain region) [31,32]. The Flt-1 and KDR/Flk-1 genes are both expressed in endothelial cells, but they have somewhat different functions. Knockout experiments showed that KDR/Flk-1 played a central role in endothelial cell proliferation and differentiation[33,34]. What's more, KDR/Flk-1 was constitutively expressed in choroid-retinal endothelial cell and up-regulated under hypoxic conditions[35], therefore, KDR/Flk-1 expression was involved in this experiment. Our data suggested that pericytes could decrease the expression of KDR/Flk-1 mRNA in RMECs, which may partially explain the mechanism of pericytes' inhibition effect on growth of RMECs. It was observed that down- regulating effect couldn't fully reverse RMECs' proliferation induced by hypoxia, which indicated that involvement of other growth factors and receptors must also be taken into account. In addition to VEGF, hypoxia can also induce increasing expression of other growth factors, among which, bFGF, TGF-β, and PDGF-β seem particularly important

because they could be produced by endothelial cells themselves, thus possessing potential autocrine activities[36,37]. As for the mechanism pericytes down-regulated KDR/Flk-1 mRNA expression, further research need to be carried out. In conclusion, the present study demonstrated that pericytes inhibited RMECs proliferation *in vitro* both under normoxia and hypoxia condition, which partially by down-regulating KDR/Flk-1 mRNA in RMECs.

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