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# Hypoxia-induced expression of vascular endothelial growth factor by retinal glial cells promotes in vitro angiogenesis

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**Abstract** To determine whether retinal glial cells (RGCs) participate in the paracrine regulation of retinal neovascularization, we investigated whether cultured RGCs synthesize and release vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) under normoxic or hypoxic conditions. Northern blot analysis demonstrated that cultured RGCs transcribed both VEGF mRNA with two molecular bands approximately 3.9 and 4.3 kilobases (kb), and bFGF mRNA with approximately 3.7 and 6.0 kb. The expression of VEGF mRNA was greatly enhanced by hypoxic cultivation (2% oxygen) when compared with normoxic cultivation (20% oxygen), while the expression of bFGF mRNA by RGCs was not significantly affected by hypoxia. The effects of RGCs-conditioned media (CM) on tritiated-thymidine incorporation and in vitro angiogenesis by retinal capillary endothelial cells (RECs) in producing the formation of capillary-like tubes in type I collagen gels, were evident in the observation that RGCs-CM harvested after hypoxic cultivation significantly enhanced tritiated-thymidine incorporation (1.9 times, $P<0.01$) and in vitro angiogenesis (2.4 times, $P<0.01$) compared with the normoxic RGCs-CM. These enhancing effects of RGCs-CM at hypoxia were suppressed by anti-VEGF neutralizing antibody. Furthermore, RECs were shown to express mRNA encoding the VEGF receptor *flt*-1 by northern blot analysis. These results suggest that VEGF expressed by RGCs under hypoxic conditions plays an integral role in the initiation and progression of retinal neovascularization in a paracrine manner.

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**Key words** Vascular endothelial growth factor · Retinal glial cells · Hypoxia · Angiogenesis · Retinal capillary endothelial cells

## Introduction

Angiogenesis is an essential and well controlled process in both physiological development and growth of mammalian organs and in pathological conditions such as the inflammation-repair process [15]. However, a sustained and excessive proliferation of blood vessels causes such disease states as proliferative diabetic retinopathy and the growth and metastasis of malignant neoplasms.

Ischaemia is a ubiquitous feature observed in various retinal vascular disorders such as diabetic retinopathy and retinal vein occlusion. In diabetic retinopathy, prolonged hyperglycaemia leads to both anatomical and functional changes in capillaries with thickening of the subendothelial basement membrane, matricial increment, the selective loss of pericytes [43] and the formation of avascular areas, resulting in tissue ischaemia/hypoxia which has been generally accepted to precede retinal neovascularization. Thus the retinal hypoxia is considered to be one of the major causes of angiogenesis in vivo. But no specific angiogenic candidate for the initiator of retinal neovascularization has been implicated conclusively.

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor [9], is a likely candidate as an angiogenic factor in retinal neovascularization because of its biological characteristics. It is a secretory mitogen [24], a specific mitogen for endothelial cells [1, 8, 11–13, 20] and a hypoxia-inducible protein [22, 37]. In diabetic retinopathy, excessive vascular permeability is frequently noticed [19] around the ischaemic retina. In addition, newly formed blood vessels are also associated with the immigration of reactive glial cells [17, 28].

Because excessive vascular permeability and endothelial proliferation have been thought to be crucial steps

leading to the initiation and progression of retinal neovascularization, we focussed on the function of VEGF and investigated whether retinal glial cells (RGCs) are a possible candidate for the production and release of VEGF.

## Materials and methods

Reagents and antibodies used in the present study are as follows. Dulbecco's modified Eagles medium (DMEM) and RPMI-1640 medium were obtained from Nissui Pharmaceuticals (Tokyo, Japan) and Gibco Laboratories (Gland Island, N.Y., USA), respectively. Fetal bovine serum (FBS) came from the Flow Laboratories (Stanmore, New South Wales, Australia). Collagenase type I was from Sigma (St. Louis, Mo., USA), type I collagen solution from Kohken (Tokyo, Japan) and tritiated-thymidine (5.0 Ci/mmol/l) from Amersham (Arlington Heights, IU., USA). Active basic fibroblast growth factor (bFGF) purified from the bovine brain and human recombinant VEGF were from R&D Systems (Minneapolis, Mass., USA) and Pepro-Teck (Rocky Hill, N.J., USA), respectively. The polyclonal antibodies against human bFGF and VEGF were purchased from Biochemical Technologies (Stouhton, Mass., USA) and Santa Cruz Biotechnology, (Calif., USA), respectively.

The biological activity of bFGF and VEGF, and their neutralization by antibodies were assessed by a tritiated-thymidine incorporation assay of the retinal capillary endothelial cells (RECs) according to the method described below. Both bFGF and VEGF enhanced tritiated-thymidine incorporation in the RECs in a dose-dependent manner, and the median effective doses ($ED_{50}$) for this enhancement were 0.35 ng/ml and 0.5 ng/ml, respectively. The 50% neutralization doses of anti-bFGF IgG for 0.35 ng/ml of bFGF and anti-VEGF IgG for 0.5 ng/ml of VEGF were 0.5 μg/ml and 0.1 μg/ml, respectively. Therefore, we used anti-bFGF IgG at 20 μg/ml and anti-VEGF IgG at 10 μg/ml for the immunological inhibition experiments of the respective growth factor.

RECs were isolated by the method previously described [38] with some modifications. Bovine eyes were dissected under sterile conditions. After incision 5 mm posterior from the limbus, the retinas were gently removed. They were then finely minced with curved scissors, and microvessels were trapped on a 74 μm nylon mesh, transferred to a petri dish containing 10 ml of 0.5% collagenase, and incubated at 37° C in a water incubator for 30 min. The digested tissue was then shifted sequentially through 105 μm and 54 μm nylon meshes. The pellet trapped on the 54 μm nylon mesh was centrifuged at 800 *g* for 5 min, and plated on gelatin coated plastic dishes containing DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were routinely split at a ratio of 1:3, and identified as endothelial cells by the exhibition of single-layered and polygonal or spindle-shaped morphology under a phase contrast microscope (Fig. 1A) and the immunocytochemical expression of von-Willebrand factor (Fig. 1B).

To isolate RGCs, the final filtrate was centrifuged at 800 *g* for 5 min, and then the pellet was plated on a gelatin coated dish. RGCs were identified by their stellate morphology under a phase contrast microscope (Fig. 1C) and the immunocytochemical expression of glial fibrillary acidic protein (not shown). Under the electron microscope, RGCs possessed a large amount of intermediate filaments in their cytoplasm (Fig. 1D).

Fibroblasts were also isolated from retrobulbar fibroconnective tissue. Bovine aortic smooth muscle cells (ASMCs) were a kind gift from Dr. A. Takei, and identified by their immunocytochemical expression of α-smooth muscle actin (not shown). The 7th to 13th passages of RECs, the 2nd or 3rd passages of RGCs and fibroblasts, and the 4th or 5th passages of ASMCs were used in the following experiments.

Confluent RGCs ($1.9\times10^4/cm^2$) supplied with 15 ml of fresh DMEM containing 3% FBS and 10 mM N-[2-hydroxyethyl]piperazine-N′-2-ethanesulphonic acid, pH 7.2, in 150 $cm^2$ culture flasks (Corning Glass Works, Corning, N.Y., USA) were cultured under either normoxic (20%) or hypoxic (2%) conditions in a humidified 5% carbon dioxide incubator at 37° C ($O_2/CO_2$ Multi Gas Incubator BL-3200, Astec, Fukuoka, Japan). RGCs cultured under hypoxic conditions for 24 h showed no apparent morphological alteration, and neither cell debris nor detachment increased after hypoxic cultivation (not shown). The conditioned media (CM) were

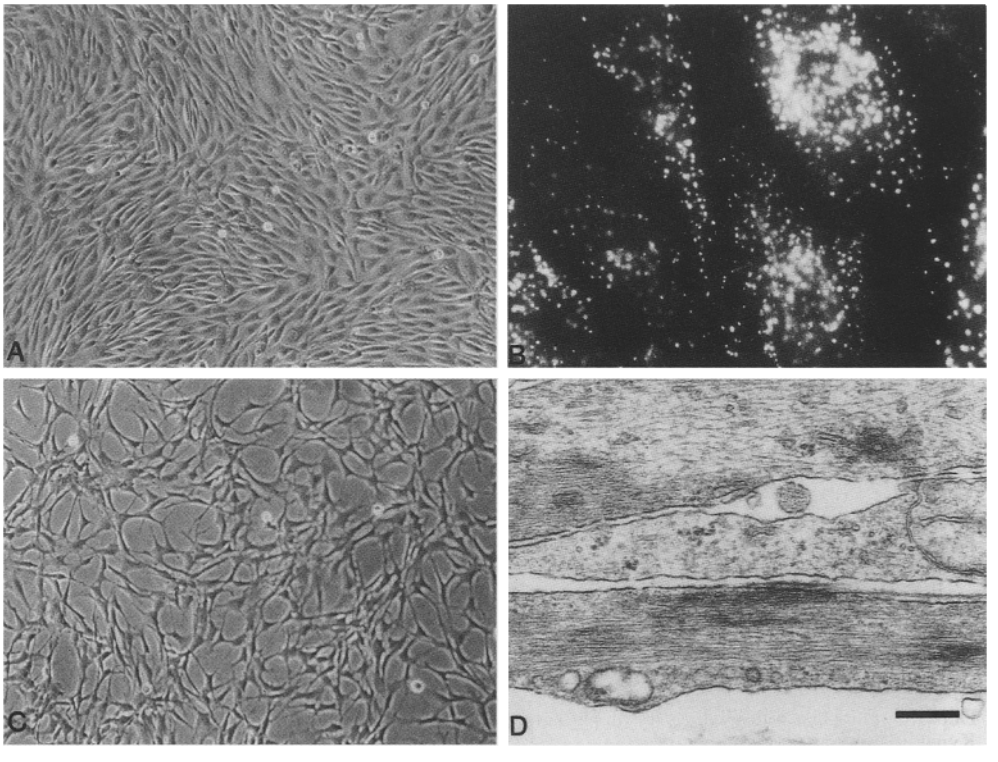



**Fig. 1A–D** Microscopic findings of cultured retinal capillary endothelial cells (RECs) and retinal glial cells (RGCs). **A** Confluent RECs cultured on a plastic dish exhibit a monolayered and spindle-shaped morphology under a phase-contrast microscope. (×12.5). **B** Immunofluorescence for von Willebrand factor. RECs show specific and characteristic granular immunofluorescence. (×250). **C** The phase-contrast microscope shows that subconfluent RGCs possess many cytoplasmic processes and frequently form networks between the cells (×25). **D** RGCs reveal numerous intermediate filaments in their cytoplasm electron microscopically (*Bar*=0.25 μm)

harvested 24 h after the cultivations, centrifuged and then sterilized by filtration through a 0.22 μm filter (Millipore, Bedford, Mass., USA). RGCs-CM were incubated overnight with non-immune IgG, anti-bFGF IgG, anti-VEGF IgG or simultaneously both anti-bFGF IgG and anti-VEGF IgG at 4° C followed by centrifugation at 6000 *g* for 30 min. The supernatants were filtrated and then used.

The effect of RGCs-CM on cell growth was evaluated by measuring the incorporation of tritiated-thymidine by RECs, ASMCs and fibroblasts according to the method of Miyazono et al. [25]. Confluent cells were detached by trypsinization and the cells were plated at a density of 1,000 cells per well, into 24-multiwell plates (Corning Glass Works) containing 0.5 ml of DMEM supplemented with 10% FBS. The cells were incubated for 6 h to allow for attachment, and then the media were exchanged with either RGCs-CM or fresh media. After incubation for 9 h, the cells were pulsed for 6 h with tritiated-thymidine (0.3 μCi/ml of medium), then washed with 0.01 M PBS (pH 7.4) and extracted with cold 5% trichloro-acetic acid. The resulting precipitates were then washed twice with ethanol-ether (volume ratio at 3:1) and solubilized with 1 N sodium hydroxide. The radioactivity was determined with a liquid scintillation counter (LSC-3500, Aloka, Tokyo, Japan). All determinations were performed in triplicate.

The collagen gels used for in vitro angiogenesis assay were prepared as previously described [33, 44]. The 0.2% collagen gels were prepared by mixing 6 ml of 0.3% type I collagen solution, with 0.9 ml of 10 times concentrate of RPMI-1640 medium, 1.1 ml of 1.6% sodium bicarbonate and 1 ml of distilled water. One hundred microlitres of solution was put on the filter of Millicell-CM inserts (12 mm in diameter, Millipore), which were placed in 12-multiwell plates (Corning Glass Works). The gels were used after gelatinization of the collagen solution in a humidified carbon dioxide incubator at 37° C for 30 min.

In this assay system, each well was composed of double chambers, consisting of an outer chamber, 12-multiwell plate, and an inner Millicell-CM chamber. In an inner chamber, RECs ($6.5 \times 10^3$) were seeded in 0.3 ml of DMEM supplemented with 10% FBS, and 1.5 ml of RGCs-CM or fresh DMEM supplemented with 3% FBS as a control was poured into an outer chamber (Fig. 2). RECs started to migrate into the type I collagen gel and began to form tubular structures beneath the confluent monolayer (Fig. 3B) after about 24 h. These structures were observed under a phase-contrast microscope (Fig. 3A), and after 3 days cultivation nine areas selected at random were photographed in each well of triplicate cultures. The length of the tubular structure was then measured with a Cosmozone 1S image analyzer (Nikon, Tokyo, Japan). The total length of branching tubes longer than 100 μm was calculated for each well and compared with the tube length produced under experimental conditions. Under the electron microscope, each organized tube was usually composed of several RECs with the formation of a junctional complex between their cytoplasmic extensions (Fig. 3C).

The cDNA probes used for the northern blot analysis were as follows: human VEGF cDNA with 0.93 kb (a gift from Dr. N. Ferrara, Genentech, San Francisco, Calif., USA) [24], human *fms*-like tyrosine kinase-1 (*flt*-1) cDNA with 2.4 kb [42], human bFGF

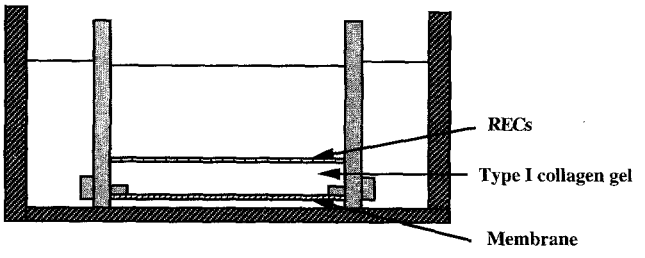



**Fig. 2** A three-dimensional model of in vitro angiogenesis. In this assay system, each well is composed of double chambers, namely, an outer chamber (12-multiwell plate) and an inner chamber (Millicell-CM). RECs are seeded on type I collagen gel in 0.1 ml of Dulbecco's modified Eagles medium containing 10% serum placed on a 0.4 μm filter in the inner chamber. Either conditioned media or a fresh medium containing 3% serum were poured into the outer chamber

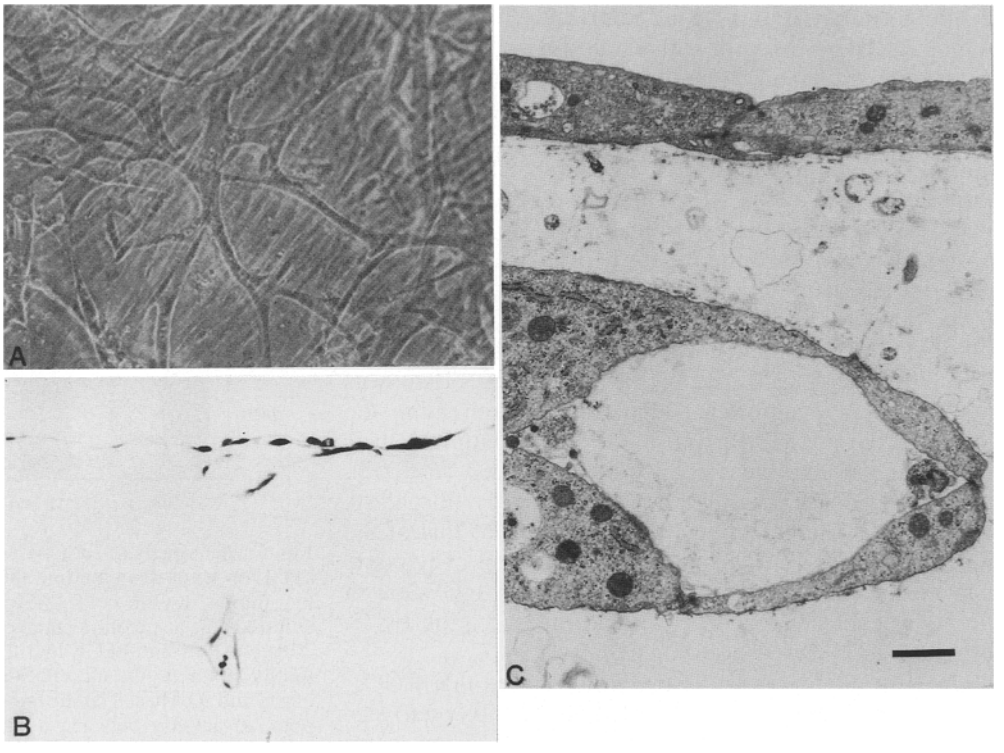



**Fig. 3A–C** Microscopy of capillary-like tubes formed by RECs. **A** Capillary-like structures formed by RECs in type I collagen gels are identified by a phase-contrast microscope. RECs form branching and anastomosing tubular structures. The focus is beneath the surface monolayer. (×25). **B** Longitudinal capillary-like tube formation by RECs just beneath the surface monolayer (Haematoxylin and eosin, ×100). **C** A tubular structure is revealed electron microscopically to consist of several endothelial cells with the formation of a junctional complex between their cytoplasmic extensions in type I collagen gel beneath the surface monolayer of RECs. (*Bar*=1.0 μm)

cDNA with 0.49 kb (a gift from Mochida Pharmaceuticals, Japan) and rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA with 0.3 kb (a gift from Dr. T. Ando, the Second Department of Internal Medicine, Kyushu University) [43]. The probes were prepared by the random primer [$\alpha$-$^{32}$P] dCTP cDNA labelling method [10].

For northern blot analysis total cellular RNA from the confluent RGCs and RECs was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method [7]. Total RNA was fractionated by electrophoresis on a 1% agarose-formaldehyde gel. The samples were transferred overnight to a nylon membrane (GeneScreen, Boston, Mass., USA) by capillary blotting and the membrane was baked at 60° C for 3 h. The blots were prehybridized for 6 h at 42° C with 50% formamide, 6×standard saline citrate (SSC), 200 µg/ml tRNA, 2×Denhardt's solution, 0.5% sodium dodecylsulphate (SDS) and 10 mM sodium phosphate, and then incubated overnight with a $^{32}$P-labelled cDNA probe. The membrane was washed once with 2×SSC and 0.1% SDS, and twice with 0.2×SSC and 0.1% SDS at 42° C. The signals were visualized by autoradiography, and the molecular weights were determined by a comparison with the RNA ladder (Gibco, Grand Island, N.Y., USA) of known molecular weights. The mRNA levels were measured by scanning using a BAS 2000 (Fuji, Tokyo, Japan), and then normalized to the mRNA level of GAPDH.

Our results were expressed as the mean±standard deviation. Statistical comparisons were made using Student's unpaired *t*-test, and a value $P<0.05$ was considered to be statistically significant.

## Results

RGCs-CM obtained under hypoxic conditions (2% oxygen) significantly stimulated the incorporation of thymidine by RECs (2.2-times of the fresh media, $P<0.01$) while the normoxic RGCs-CM did not (Fig. 4). By pretreating the hypoxic RGCs-CM with anti-bFGF IgG (20 µg/ml), this enhancing effect on thymidine incorporation of RECs was reduced (38%,$P<0.02$). Even after this pretreatment however, the residual enhancing activity of thymidine incorporation was still significant (1.5 times of the normoxic RGCs-CM, $P<0.01$). By pretreating the hypoxic RGCs-CM with anti-VEGF IgG (10 µg/ml), the enhancing effect on thymidine incorporation by RECs was almost totally abrogated (95%, $P<0.01$). Simultaneous pretreatment of the hypoxic RGCs-CM with both anti-bFGF IgG and anti-VEGF IgG reduced the incorporation of thymidine by RECs to a lower level when compared with that of the fresh media ($P<0.02$).

We also examined the effects of RGCs-CM on thymidine incorporation by fibroblasts and ASMCs. RGCs-CM obtained under a hypoxic condition (2% oxygen) mildly stimulated the incorporation of thymidine by both fibroblasts and ASMCs, but this enhancing effect was not statistically significant (Fig. 5). By pretreating the hypoxic RGCs-CM with anti-bFGF IgG (20 µg/ml), their promoting effects were significantly reduced ($P<0.02$), and the levels of thymidine incorporation by fibroblasts and ASMCs reached almost the same level as that of the normoxic RGCs-CM. However, pretreatment of hypoxic and normoxic RGCs-CM with anti-VEGF IgG showed no significant change in thymidine incorporation by fibroblasts or ASMCs (not shown).

In vitro angiogenesis assay demonstrated that RGCs-CM obtained under hypoxic conditions (2% oxygen) significantly enhanced in vitro angiogenesis when compared with the fresh media and normoxic RGCs-CM

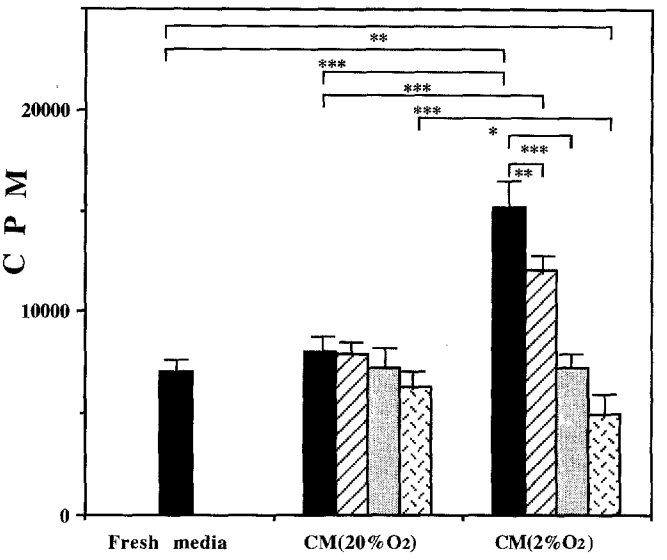



**Fig. 4** The effects of conditioned medium from the RGCs (RGCs-CM) and neutralizing antibody against basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) on tritiated-thymidine incorporation by RECs. RECs were cultured in a medium pretreated with non-immune IgG (■, 20 µg/ml), anti-bFGF IgG (▨, 20 µg/ml), anti-VEGF IgG (▩, 10 µg/ml) or simultaneously with both anti-bFGF IgG and anti-VEGF IgG (▧). Hypoxic RGCs-CM enhances the incorporation of tritiated-thymidine by RECs 1.9 times of the normoxic RGCs-CM ($P<0.01$) and 2.2 times of the fresh media ($P<0.01$). The pretreatment of the hypoxic RGCs-CM with anti-VEGF IgG inhibits this enhancing effect on tritiated-thymidine incorporation more dominantly (95%,$P<0.01$) than with anti-bFGF IgG (38%, $P<0.02$). Each column represents the mean ±standard deviation of experiments carried out in triplicate. (*CPM* counts per minute, $O_2$ oxygen, * $P<0.05$, ** $P<0.02$, *** $P<0.01$)

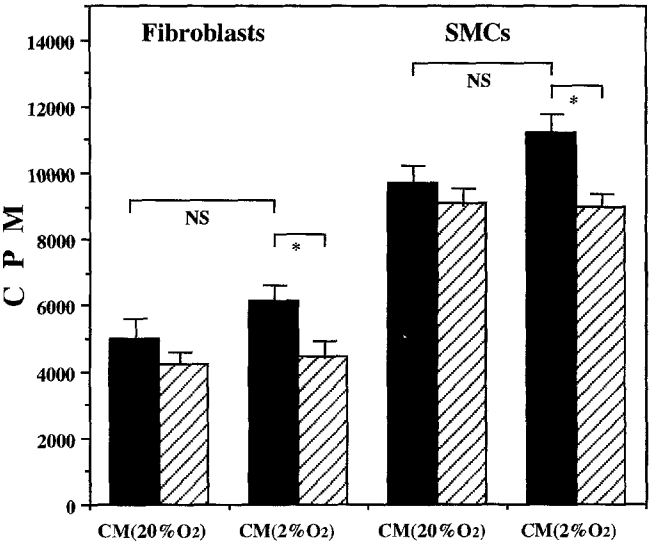



**Fig. 5** The effects of RGCs-CM and neutralizing antibody against bFGF on tritiated-thymidine incorporation by fibroblasts and aortic smooth muscle cells (ASMCs). Fibroblasts or ASMCs were cultured in a medium pretreated with non-immune IgG (■, 20 µg/ml), or anti-bFGF IgG (▨, 20 µg/ml). Hypoxic RGCs-CM mildly stimulate the incorporation of tritiated-thymidine by fibroblasts and ASMCs. The difference of the tritiated-thymidine incorporation activity between normoxic and hypoxic RGCs-CM becomes non-significant after the preincubation of RGCs-CM with anti-bFGF IgG. (* $P<0.02$, *NS* not significant)

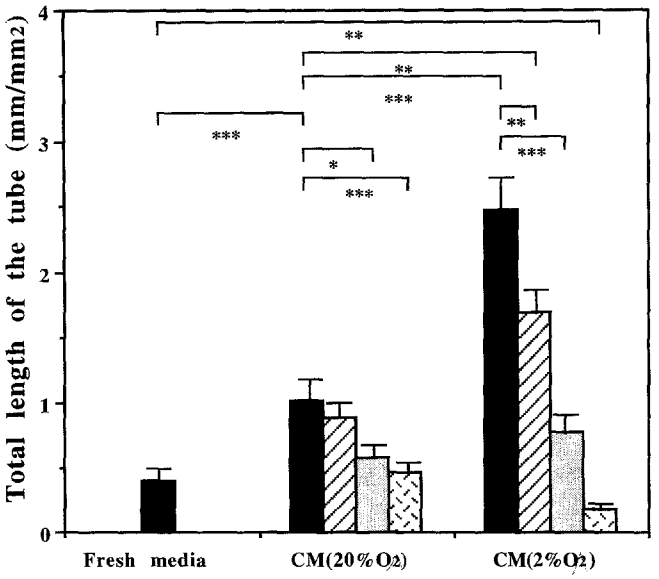



**Fig. 6** The effects of RGCs-CM and neutralizing antibodies against bFGF or VEGF on in vitro angiogenesis. RECs were cultured in a medium pretreated with non-immune IgG (■, 20 μg/ml), anti-bFGF IgG (▨, 20 μg/ml), anti-VEGF IgG (▩, 10 μg/ml) or simultaneously with both anti-bFGF IgG and anti-VEGF IgG (▽). Hypoxic RGCs-CM significantly enhances the formation of capillary-like tubes by RECs ($P<0.01$). This promoting effect is 2.4 times that of the normoxic RGCs-CM, and 6.2 times that of the fresh media. The enhancing effect of RGCs-CM on in vitro angiogenesis is abrogated more dominantly by the preincubation of RGCs-CM with anti-VEGF IgG (97%, $P<0.01$) than with anti-bFGF IgG (38%, $P<0.02$) (* $P<0.05$, ** $P<0.02$, *** $P<0.01$)

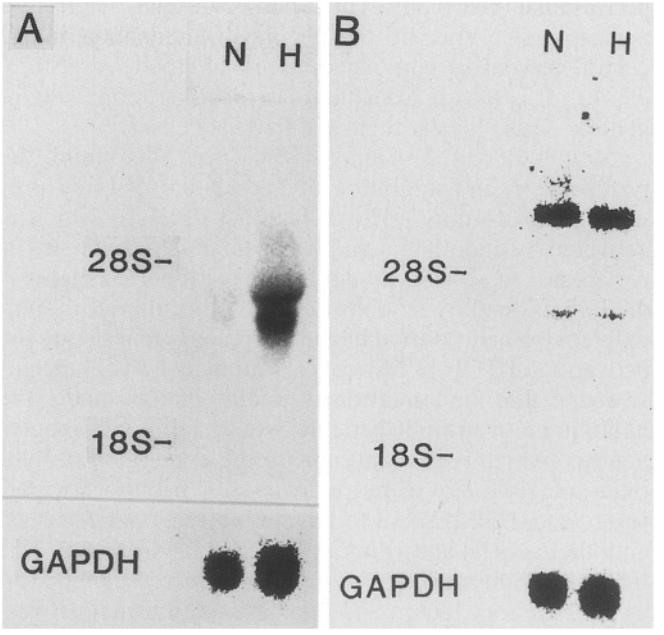



**Fig. 7A, B** Northern blot analysis for VEGF and bFGF gene expression by RGCs. **A** RGCs cultured under normoxic (lane *N*) or hypoxic (lane *H*) conditions express two VEGF mRNA transcripts of approximately 3.9 and 4.3 kb. In addition, the hypoxic cultivation markedly enhances the expression of both transcripts by RGCs.The positions of rRNA are indicated on the left. **B** The cultured RGCs express two bFGF mRNA transcripts of approximately 6.0 and 3.7 kb. The expression of bFGF mRNA by RGCs showed no significant change after hypoxic cultivation.

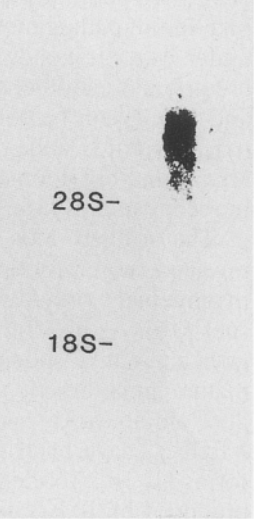
(20% oxygen). The promoting effect of hypoxic RGCs-CM on in vitro angiogenesis was 2.4-times that of the normoxic RGCs-CM ($P<0.01$), and 6.2-times that of fresh media ($P<0.01$; Fig. 6).

The enhancing effect of hypoxic RGCs-CM on tube-forming activity was almost completely abrogated by pretreating the RGCs-CM with anti-VEGF IgG (97%, $P<0.01$) when compared with that pretreated with anti-bFGF IgG (38%, $P<0.02$). By the simultaneous pretreatment of hypoxic RGCs-CM with both anti-bFGF IgG and anti-VEGF IgG, the tube-forming activity of the RECs was reduced to a lower level when compared with the fresh media ($P<0.02$).

Northern blot analysis showed that the total RNA obtained from RGCs cultured under normoxic or hypoxic conditions identified two transcripts of approximately 3.9 and 4.3 kb with a $^{32}$P-labelled VEGF cDNA probe (Fig. 7A). The cultured RGCs also showed two transcripts of approximately 6.0 and 3.7 kb with a $^{32}$P-labelled bFGF cDNA probe (Fig. 7B). The expression of VEGF mRNA was greatly increased after hypoxic cultivation, whereas the expression of bFGF mRNA showed no significant change.

In addition, northern blot analysis for *flt*-1 gene expression by RECs cultured under normoxic conditions revealed an approximately 8.5 kb mRNA (Fig. 8).

**Fig. 8** The *flt*-1 gene expression by cultured RECs. Fifteen micrograms of total RNA isolated from RECs cultured under normoxic conditions was hybridized with a $^{32}$P-labelled *flt*-1 cDNA probe and a band of approximately 8.5 kb was revealed




## Discussion

Neovascularization is not regulated solely by the presence or absence of stimulatory and inhibitory factor(s) but by the maintenance of a balance between factors [36], through an autocrine or paracrine mechanism mediated by smooth muscle cells, pericytes, macrophages, mast cells and other cell types. The retinal capillaries are composed of endothelial cells and pericytes, largely ensheathed by

perivascular glial cells. This endothelial-glial interaction participates in specific endothelial differentiation in the central nervous system including retinal blood vessels [23, 29, 40]. It is thus reasonable to assume that retinal angiogenesis is also locally regulated by glial cells.

Sato et al. [34, 35] reported that pericytes inhibit the proliferation and migration of endothelial cells by producing transforming growth factor-β (TGF-β) which is activated by endothelial and pericytic co-existence in vitro. Sakuda et al. [33], in contrast have reported that media conditioned by smooth muscle cells cultured in hypoxic enviroments stimulates in vitro angiogenesis via the activation of TGF-β. Moreover, Murata et al. [27] recently found that the conditioned media obtained under the hypoxic cultivation of pericytes stimulate in vitro angiogenesis, which is probably due to the expression of hypoxia-inducible VEGF by pericytes [2]. In this way, pericytes and TGF-β seem to have a bidirectional effect on endothelial cells according to the local environment. It is likely that a loss of pericytes causes a decrease of TGF-β as an inhibitory factor, and thus promotes retinal neovascularization. Nevertheless, it remains unclear to what extent VEGF derived from pericytes can initiate and support retinal neovascularization in vivo, because it is known that pericytes degenerate selectively and frequently disappear from retinal capillaries in the early stage of diabetic retinopathy [43].

In this study, we demonstrated that cultured RGCs synthesized and released VEGF into the conditioned medium, which induced endothelial specific proliferation and in vitro angiogenesis. These findings suggest that RGCs are another possible source of VEGF and participate in the pathogenesis of the retinal neovascularization under hypoxic conditions. Unlike bFGF, VEGF is a secretory and endothelial specific mitogen. But both bFGF and VEGF can be colocalized in or adjacent to the hypoxic tissue [3] and, further, a synergistic effect of both VEGF and bFGF on endothelial cells has been suggested in recent studies [16, 30].

The northern blot analysis for VEGF gene expression revealed two transcripts of approximately 3.9 and 4.3 kb in agreement with VEGF transcripts observed in other tissues [16]. As previously described, VEGF mRNA undergoes alternative splicing leading to the production of four mature homodimeric proteins with either 121, 165, 189, or 206 amino acids as a monomer [18]. $VEGF_{121}$ and $VEGF_{165}$ have been reported to be the predominant isoforms [1, 16]. To identify the molecular species of VEGF produced by RGCs, we reverse-transcribed the RNA and amplified the cDNA by polymerase chain reaction, and identified the two transcripts to correspond to the mRNA encoding human $VEGF_{121}$ and $VEGF_{165}$.

Furthermore, we also demonstrated that hypoxia markedly stimulated RGCs to express VEGF mRNA. Possible mechanisms proposed for enhanced gene expression in hypoxic conditions include the activated transcription of genes encoding factors such as erythropoietin and interleukin-8 [4, 21] and the increased stability of mRNA [6]. However, the precise mechanism(s) regulating VEGF gene expression by RGCs, especially in a hypoxic state, remains to be investigated.

In addition, although hypoxia is a potent inducer of VEGF gene expression, its over-expression is also reported in the absence of hypoxic conditions, as in cerebellar haemangioblastoma [26, 32] and adenocarcinomas [5]. It is therefore possible that the expression of VEGF gene is not only regulated by the oxygen concentration but also other modulators such as platelet-derived growth factor (PDGF) [14].

The northern blot analysis for bFGF gene expression showed that RGCs expressed two transcripts of bFGF mRNA under normoxic conditions, but this expression showed no significant change after hypoxic cultivation. While cell debris was not apparently increased after hypoxic cultivation of RGCs in this study, the release of bFGF into the CM as a result of the sublethal hypoxic effect on RGCs might relate to the promotion of thymidine incorporation and in vitro angiogenesis by RECs.

We also ascertained *flt*-1 gene expression by RECs, which is one of the VEGF receptors and a member of PDGF receptor family [42]. This suggests that VEGF expressed by RGCs functions through the VEGF-*flt* interaction in a paracrine manner on retinal capillary endothelial cells. It is known that the expression of *flt*-1 by capillary endothelial cells is also up-regulated around the tumour tissue especially around the necrotic foci [31]. As capillary endothelial cells migrating into hypoxic areas such as the central region of tumours and ischaemic retina are exposed to hypoxic conditions, we investigated the effect of hypoxia on *flt*-1 gene expression by RECs, and no apparent alteration was detected by hypoxic cultivation (not shown). Some undefined factor(s) produced by the cells surrounding endothelial cells in the hypoxic microenvironment may act as up-regulating modulator(s) of *flt*-1 gene expression.

Simorre-Pinatel et al. [38] recently found that RECs synthesize VEGF and stimulate RECs through an autocrine pathway. Therefore, retinal neovascularization seems to be regulated by VEGF function via both an autocrine and paracrine manner, in association with RGCs, pericytes and other retinal cells.

Our findings indicate that VEGF expressed by RGCs may play an integral role in the initiation and progression of retinal neovascularization together with bFGF. The possible synergistic effect of VEGF and bFGF on RECs was unclear in this study. This is the first confirmation that RGCs are one of the potent angiogenesis inducing candidates in ocular tissues, acting by the production and release of VEGF under hypoxic conditions.

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## References


1. Adamis AP, Shima DT, Yeo K-T, Yeo T-K, Brown LF, Berse B, D'Amore PA, Folkman J (1993) Synthesis and secretion of vascular permeability factor/vascular endothelial growth factor by human retinal pigment epithelial cells. Biochem Biophys Res Commun 193:631–638
2. Aiello LP, Ferrara N, King GL (1994) Hypoxic regulation of vascular endothelial growth factor: Characterization in retinal microvascular pericytes and pigment epithelial cells (abstract). Invest Ophthalmol Vis Sci 5:1868
3. Alvarez JA, Baird A, Tatum A, Daucher J, Chorsky R, Gonzalez AM, Stopa EG (1992) Localization of basic fibroblast growth factor and vascular endothelial growth factor in human glial neoplasms. Mod Pathol 5:303–307
4. Blanchard KL, Acquaviva AM, Galson DL, Bunn HF (1992) Hypoxic induction of the human erythropoietin gene: Cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. Mol Cell Biol 12:5373–5385
5. Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Senger DR, Dvorak HF (1993) Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. Cancer Res 53:4727–4735
6. Carter BZ, Malter JS (1991) Biology of disease. Regulation of mRNA stability and its relevance to desease. Lab Invest 65: 610–621
7. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
8. Connolly DT (1991) Vascular permeability factor: A unique regulator of blood vessel function. J Cell Biochem 47:219–223
9. Connolly DT, Olander JV, Heuvelman D, Nelson R, Monsell R, Siegel N, Haymore BL, Leimgruber R, Feder J (1989) Human vascular permeability factor: Isolation from U937 cells. J Biol Chem 264:20017–20024
10. Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13
11. Ferrara N, Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 161:851–858
12. Ferrara N, Houck KA, Jakeman LB, Winer J, Leung DW (1991) The vascular endothelial growth factor family of polypeptides. J Cell Biochem 47:211–218
13. Ferrara N, Houck K, Jakeman L, Leung DW (1992) Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocr Rev 13:18–32
14. Finkenzeller G, Marme D, Weich HA, Hug H (1992) Platelet-derived growth factor-induced transcription of vascular endothelial growth factor gene is mediated by protein kinase C. Cancer Res 52:4821–4823
15. Folkman J, Shing Y (1992) Angiogenesis. J Biol Chem 267: 10931–10934
16. Goto F, Goto K, Weindel K, Folkman J (1993) Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation on bovine capillary endothelial cells within collagen gels. Lab Invest 69:508–517
17. Hosoda Y, Okada M, Matsumura M, Ogino N, Honda Y, Nagai Y (1993) Epiretinal membrane of proliferative diabetic retinopathy: An immunohistochemical study. Ophthalmic Res 25: 289–294
18. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW (1991) The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol 5:1806–1814
19. Ishibashi T, Tanaka K, Taniguchi Y (1980) Disruption of blood-retinal barrier in experimental diabetic rats: An electron microscopic study. Exp Eye Res 30:401–410
20. Jakeman LB, Winer J, Bennett GL, Altar A, Ferrara N (1992) Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. J Clin Invest 89: 244–253
21. Karakurum M, Shreeniwas R, Chen J, Pinsky D, Yan S-D, Anderson M, Sunouchi K, Major J, Hamilton T, Kuwabara K, Rot A, Nowygrod R, Stern D (1994) Hypoxic induction of interleukin-8 gene expression in human endothelial cells. J Clin Invest 93:1564–1570
22. Ladoux A, Frelin C (1993) Hypoxia is a strong inducer of vascular endothelial growth factor mRNA expression in the heart. Biochem Biophys Res Commun 195:1005–1010
23. Laterra J, Guerin C, Goldstein GW (1990) Astrocytes induce neural microvascular endothelial cells to form capillary-like structures in vitro. J Cell Physiol 144:204–215
24. Leung DW, Cachianes G, Kuang W-J, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306–1309
25. Miyazono K, Okabe T, Ishibashi S, Urabe A, Takaku F (1985) A platelet factor stimulating the proliferation of vascular endothelial cells: Partial purification and characterization. Exp Cell Res159:487–494
26. Morii K, Tanaka R, Washiyama K, Kumanishi T, Kuwano R (1993) Expression of vascular endothelial growth factor in capillary hemangioblastoma. Biochem Biophys Res Commun 194:749–755
27. Murata T, Ishibashi T, Inomata H, Sueishi K (1994) Media conditioned by coculture of pericytes and endothelial cells under a hypoxic state stimulate in vitro angiogenesis. Ophthalmic Res 26:23–31
28. Nork TM, Wallow IHL, Sramek SJ, Anderson G (1987) Müller's cell involvement in proliferative diabetic retinopathy. Arch Ophthalmol 105:1424–1429
29. Penfold PL, Provis JM, Madigan MC, Driel D van, Billson FA (1990) Angiogenesis in normal human retinal development: the involvement of astrocytes and macrophages. Graefes Arch Clin Exp Ophthalmol 228:255–263
30. Pepper MS, Ferrara N, Orci L, Montesano R (1992) Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. Biochem Biophys Res Commun 189:824–831
31. Plate KH, Breier G, Weich HA, Risau W (1992) Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 359:845–848
32. Plate K, Wizigmann-Voos S, Breier G, Risau W (1994) Evidence for paracrine growth regulatory mechanism in human hemangioblastomas (abstract). Clin Neuropathol 13: 264
33. Sakuda H, Nakashima Y, Kuriyama S, Sueishi K (1992) Media conditioned by smooth muscle cells cultured in a variety of hypoxic environments stimulates in vitro angiogenesis: A relationship to transforming growth factor-β1. Am J Pathol 141: 1507–1516
34. Sato Y, Rifkin DB (1989) Inhibition of endothelial cell movement by pericytes and smooth muscle cells: Activation of a latent transforming growth factor-β1-like molecule by plasmin during co-culture. J Cell Biol 109:309–315
35. Sato Y, Tsuboi R, Lyons R, Moses H, Rifkin DB (1990) Characterization of the activation of latent TGF-β by co-cultures of endothelial cells and pericytes or smooth muscle cells: A self-regulating system. J Cell Biol 111:757–763
36. Schultz GS, Grant MB (1991) Neovascular growth factors. Eye 170–180
37. Shweiki D, Itin A, Soffer D, Keshet E (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359:843–845
38. Simorre-Pinatel V, Guerrin M, Chollet P, Penary M, Clamens S, Malecaze F, Plouet J (1994) Vasculotropin-VEGF stimulat-

es retinal capillary endothelial cells through an autocrine pathways. Invest Ophthalmol Vis Sci 35:3393–3400
39. Su T, Gillies MC (1992) A simple method for the in vitro culture of human retinal capillary endothelial cells. Invest Ophthalmol Vis Sci 33:2809–2813
40. Tagami M, Yamagata K, Fujino H, Kubota A, Nara Y, Yamori Y (1992) Morphological differentiation of endothelial cells cocultured with astrocytes on type-I or type-IV collagen. Cell Tissue Res 268:225–232
41. Terada Y, Tomita K, Nonoguchi H, Marumo F (1992) Different localization of two types of endothelin receptor mRNA in microdissected rat nephron segments using reverse transcription and polymerase chain reaction assay. J Clin Invest 90:107–112
42. Vries C de, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT (1992) The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255:989–991
43. Yanoff M (1966) Diabetic retinopathy. New Engl J Med 274: 1344–1349
44. Yasunaga C, Naskashima Y, Sueishi K (1989) A role of fibrinolytic activity in angiogenesis.Quantitative assay using in vitro method. Lab Invest 61:698–704