Retinal Glial Cells Stimulate Microvascular Pericyte Proliferation Via Fibroblast Growth Factor and Platelet-derived Growth Factor In Vitro

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Purpose: To investigate whether retinal glial cells (RGCs), which are believed to play an important role in the development and maintenance of microvessels, stimulate the proliferation of retinal bovine microvascular pericytes, an essential component of the vessels.

Methods: Conditioned medium (CM) was collected from a primary culture of RGC obtained from chick embryonic retina. The cell number was assayed after stimulation by RGC-CM. Also, by neutralizing antibody and reverse transcription polymerase chain reaction (RT-PCR), we tried to identify which factor of the RGCs contributes to the pericyte stimulation.

Results: Pericyte proliferation was stimulated by RGC-CM in a dose-dependent manner. Platelet-derived growth factor-BB (PDGF-BB), acidic fibroblast growth factor (aFGF), and basic fibroblast growth factor (bFGF) stimulated pericyte proliferation; however, PDGF-AA, transforming growth factor-β2 (TGF-β2), and vascular endothelial growth factor (VEGF) did not. The RGC-CM-dependent stimulative effect was blocked, in part, by the neutralizing antibodies for aFGF, bFGF, and PDGF. A mixture of these three antibodies completely blocked the stimulation. RT-PCR revealed that RNA for aFGF, bFGF, and TGF-β2 were expressed in RGCs.

Conclusions: Pericyte growth is stimulated in vitro by RGC-CM through aFGF, bFGF, PDGF-BB, at least in part. This finding suggests that RGCs may modulate in vivo pericyte cell growth through these three growth factors. **Jpn J Ophthalmol 2002;46:413–418** © 2002 Japanese Ophthalmological Society

Key Words: Fibroblast growth factor, platelet-derived growth factor, retinal glial cell, retinal microvascular pericyte.

Introduction

Retinal microvessels are composed of retinal microvascular endothelial cells and pericytes. The proliferation of these two types of cells is modulated by transforming growth factor (TGF)-β, endothelin-1.[1,2] Of these two types of cells, retinal microvascular pericytes are believed to play a key role in the maintenance of homeostasis of the retinal microvessels. This conclusion is based on the fact that pericytes have been reported to modulate vascular endothelial cells and that pericyte loss is an initiating event in the angiogenesis of proliferative diabetic retinopathy.[3]

Retinal glial cells (RGCs) also appear to play a key role in the preservation of and/or the development of retinal microvessels because, histologically, they are often found in close proximity to retinal capillaries. This hypothesis is based on the following observations: (1) administration of nerve growth factor, which is generally thought to stimulate retinal ganglion cells and retinal Müller cells, but not pericytes, consequently suppresses pericyte loss in experimental diabetic rats;[4] (2) it has been shown that retinal vessels develop with the guidance of the migration of astrocytes, which have functional similarities to retinal glial cells.[5] Thus, an investigation of the relationship between retinal pericytes and retinal

glial cells seems important for a better understanding, not only of the pathogenesis of diabetic retinopathy, but also of the mechanism of the development of the retinal vessels whose failure leads to retinopathy of prematurity.

This study investigated the issue of whether RGCs modulate retinal microvascular pericyte proliferation in vitro. In addition, we also investigated how RGCs modulate the proliferation of retinal pericytes.

Materials and Methods

Cells

Retinal microvascular pericytes were obtained from fresh bovine eyes according to the method described previously.[6] The cells were maintained in a mixture of Dulbecco's modified Eagle's medium (DMEM; Nikken Bio Medical Laboratory, Tokyo) and Ham F-12 medium (Nikken) (1:1 v/v) supplemented with 15% fetal bovine serum (FBS; Gibco BRL, Rockville, MD, USA). Only the cells that had three or four passages were used experimentally.

Chick embryonic RGCs also were obtained as described previously.[7] Briefly, the retina was dissected free of the sclera-choroid-retinal pigmented epithelium from 10-day embryonic chicks. The cells were grown in DMEM and Ham F-12 (1:1, v/v) with 15% FBS as well. By 7 days, a monolayer of RGC cells was developed. The overlying neuronal network was completely removed mechanically.

Conditioned Medium Preparation

When the primary culture of RGCs reached confluence, the media was replaced with DMEM containing 1% FBS, and incubated at 37°C for 48 hours. The collected conditioned medium (CM) was centrifuged at 1000 × g for 10 minutes at 4°C (Tomy Seiko, Tokyo) in order to remove the precipitates and/or cell debris. The CM was stored at −70°C until required for use. Control CM was prepared in the similar way from the media but without cells. Briefly, DMEM with 1% FBS, but without RGCs, was incubated at 37°C for 48 hours, and frozen at −70°C after centrifugation.

Cell Proliferation Assay

Bovine retinal pericytes were harvested in non-coated 96-well plates (Nunc, Roskilde, Denmark) at a density of 1000 cells/well in DMEM with 15% FBS, and incubated at 37°C with 5% CO_2 for 24 hours. Then the serum was starved with 1% FBS for the next 18 hours, and thereafter pericytes were cultured under various conditions for 3 or 6 days. The medium was changed at 3-day intervals.

The number of pericytes was evaluated by MTT assay. Briefly, 10 μL of 10 mg/mL MTT solution [3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] was added to the media, and the cells were incubated at 37°C for the next 4 hours. After aspirating the culture media, 100 μL of dimethylsulfoxide was added in order to lyse the cells. The absorbance of the cell lysate was measured at 570 nm in a plate reader (Model 450 Microplate Reader; Bio-Rad Laboratories, Richmond, CA, USA) using a 620-nm filter as a reference. The number of pericytes and the absorbance at 570 nm were in direct proportion at cell densities of up to 10,000 cells per well (data not shown). The assays were triplicated, and the mean number of pericytes as well as SD were obtained.

RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated with the Isogen Kit (Nippon Gene, Tokyo) following the manufacturer's instructions. Purification of total RNA using DNase I treatment was performed with the Atlas Pure Total RNA Isolation Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. RNA concentrations were calculated from the absorbance at 260 nm.

First-strand cDNA synthesis was performed with a first-strand cDNA synthesis kit (Advantage, Clontech), oligonucleotide (dT) (Gibco BRL), and reverse transcriptase (SuperScript II; Gibco BRL). RT reactions were performed under RNase-free conditions. The 10 μg total RNA and dT were heat denatured at 70°C for 10 minutes and chilled on ice before the RT reaction. A 25-μL volume containing 5 μL of 5 × first strand buffer, 1 μL of 10 × dNTP, 2.5 μL of dithiothreitol (100 mM), 0.5 μL RNase inhibitor, and 1 μL of SuperScript II reverse transcriptase were added to the RNA and incubated for 1 hour at 37°C. The reaction was terminated by incubation at 70°C for 20 minutes.

Oligonucleotide primers were described previously[8] for basic fibroblast growth factor (bFGF) (5′ GATCCGCACATCAAACTGC 3′ and 5′ GAT ACGTTTCTGTCCAGGTCC 3′) and transforming growth factor β2 (TGFβ2) (5′AGGAATGTGCAG GATAATT3′ and 5′ ATTTTGGGTGTTTTGC CAA 3′), respectively. They were designed to produce a 270-base pair (bp) bFGF fragment and a 269-bp TGF-β2 fragment, respectively. (Primers for bFGF amplified a PCR product of 270 bp, which cor-

responded to bases 432–701 of the sequence, while primers for TGF-β2 amplified a PCR product of 269 bp, which corresponded to bases 6452-6722 of the sequence.) Primers for acidic fibroblast growth factor (aFGF) were designed on the basis of published sequences of chicken aFGF.[9] Primers for aFGF amplified a PCR product of 261 bp corresponding to bases 341–601 of the sequence. The sequence of the forward primer for aFGF was 5′ AGCCCAAACTC CTGTACTGCA 3′, and the reverse primer, 5′ GTC TGCGTGCTTTTTGGAGA 3′.

PCR amplifications were performed as described previously.[8] All PCR amplifications started with denaturation at 94°C for 5 minutes and ended with a final elongation at 72°C for 5 minutes. The parameters for PCR amplifications were as follows: bFGF: 30 cycles of denaturation at 94°C for 30 seconds, annealing at step of 65°C for 1 minute, and extension at 72°C for 1 minute; TGF-β2: 94°C denaturation step for 30 seconds, followed by five cycles in which the initial annealing temperature of 65°C was reduced by 1°C per cycle, then 30 cycles of denaturation at 94°C for 5 minutes, annealing at 61°C for 1 minute, and extension at 72°C for 1 minute; aFGF: 35 cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 1 minute, and extension at 72°C for 1 minute. PCR products were analyzed on standardized 3% agarose gel and stained with ethidium bromide.

Reagents

Growth factors and neutralizing antibodies used in the series of experiments were as follows: Recombinant human (RH) aFGF, bFGF, Platelet-derived growth factor (PDGF)-AA, and PDGF-BB were purchased from R & D systems (Minneapolis, MN, USA), RH vascular endothelial growth factor (VEGF) and RH TGF-β2 from Genzyme Diagnostics (Cambridge, MA, USA).

Anti-aFGF neutralizing polyclonal rabbit IgG was purchased from R & D systems, anti-bovine bFGF neutralizing mouse monoclonal antibody from Upstate Biotechnology (Lake Placid, NY, USA), and anti-human PDGF neutralizing polyclonal antibody from Collaborative Biochemical Products (Bedford, MA, USA). This antibody blocks both the PDGF-A and PDGF-B chains, according to the manufacturer's instructions. Normal rabbit IgG was obtained from BioPur AG (Bubendorf, Switzerland) and used as control.

Results

Retinal microvascular pericyte cell growth in various concentrations of RGC-CM is shown in Fig-
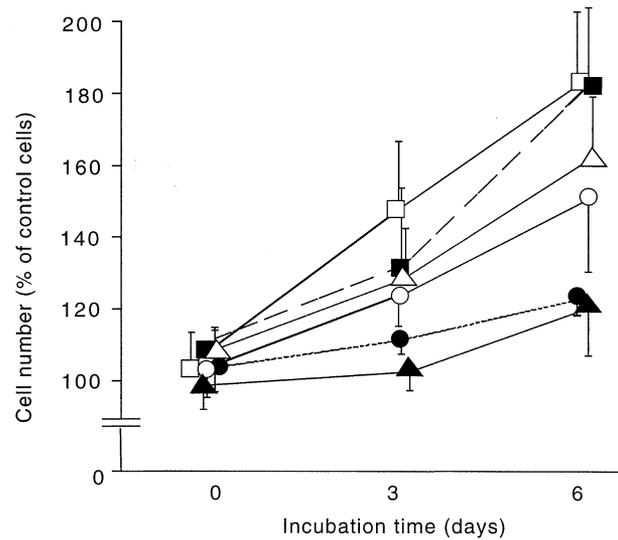
Figure 1. Retinal glial cell conditioned medium stimulates pericyte cell growth. Cells were incubated for 3 or 6 days in Dulbecco's modified Eagles's medium with 1% fetal bovine serum with retinal glial cell (RGC) conditioned medium (CM) or 50% of control CM. RGC-CM was added to increase the concentration as indicated (10–50%; v/v). The cell number for each experiment was determined and is shown as the percentage of the number of control cells that had been grown in the media with control CM. The RGC-CM stimulated pericyte growth in a dose-dependent manner. The assay was triplicated, and the data indicates mean ± SD. These are representative data from three independent experiments. □: 50% RGC-CM, ■: 40% RGC-CM, △: 30% RGC-CM, ○: 20% RGC-CM, ●: 10% RGC-CM, ▲: Control.

ure 1. The cell growth was stimulated by RGC-CM in a dose-dependent manner. A 50% mixture of RGC-CM has been shown in this study to stimulate cell growth by more than 80% over the control after 6 days.

The effect of various growth factors on pericyte growth is shown in Figure 2. Cell number was measured 3 days after stimulation with various growth factors. Pericyte growth was significantly increased by stimulation of aFGF, bFGF, and PDGF-BB in a dose-dependent manner. It is also noteworthy that VEGF, TGFβ2, and PDGF-AA did not show a prominent stimulative effect even at a concentration of 10 ng/mL.

The blocking effects of the neutralizing antibody for aFGF, bFGF, and PDGF-BB on RGC-CM stimulation are shown in Figure 3. All three neutralizing antibodies showed an inhibitory effect on cell growth stimulation. The maximum amount of the antibodies used here (10 μg/mL for anti-aFGF antibody, 10 μg/mL for anti-bFGF, and 100 μg/mL for anti-PDGF)
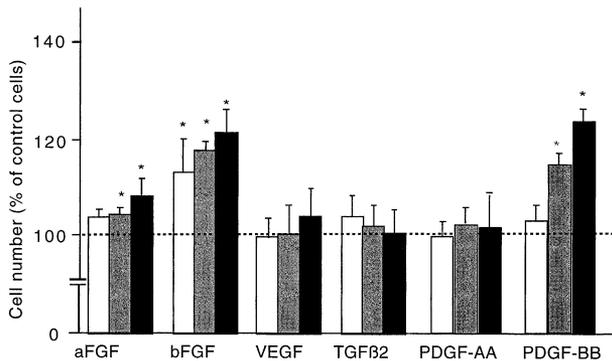
Figure 2. Human recombinant acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF)-BB stimulate pericyte cell growth. Cells were grown for 3 days with various types of growth factors including aFGF, bFGF, vascular endothelial growth factor (VEGF), transforming growth factor-β2 (TGFβ2), PDGF-AA, and PDGF-BB at concentrations of 0.1 ng/mL (open bar), 1 ng/mL (gray bar), or 10 ng/mL (closed bar), respectively. Thereafter, the cell number was determined by MTT assay and the data is shown as a percentage of the number of control cells that had been grown in the control conditioned medium without growth factor. Cell growth was stimulated by aFGF, bFGF, and PDGF-BB, but not by TGF-β2, VEGF, and PDGF-AA. These stimulative effects were dose-dependent. The assay was triplicated, and the data indicates mean ± SD. This is representative data from three independent experiments. *$P < .05$ compared to control by unpaired t-test.

were capable of blocking the cell growth from 10 ng/mL of aFGF, 10 ng/mL of bFGF, and 10 ng/mL of PDGF-BB, respectively (data not shown). The percentage of inhibition was up to 30% for aFGF, 50% for bFGF, and 70% for PDGF at concentrations of 10 µg/mL of antibody for aFGF, and bFGF, and 100 µg/mL of antibody for PDGF. In summary, aFGF, bFGF, and PDGF seem to be involved in RGC-CM-dependent pericyte stimulation.

The blocking effect by a mixture of neutralizing antibody for aFGF (10 µg/mL), bFGF (10 µg/mL), and PDGF (100 µg/mL) is shown in Figure 4. The cell growth stimulation by RGC-CM was blocked by more than 90% by the mixture of these three neutralizing antibodies (anti-aFGF, anti-bFGF, and anti-PDGF). Thus, the stimulating effect of RGC-CM seems to be dependent mostly on these growth factors, aFGF, bFGF, and PDGF.

In order to verify the expression of aFGF, bFGF, and TGFβ2 in cultured RGCs, RT-PCR was performed. The cDNA products from the RNA were found in the 3% agarose gel at the expected size (Figure 5). In summary, RNA for aFGF, bFGF, and TGFβ2 were expressed in the cultured RGCs.
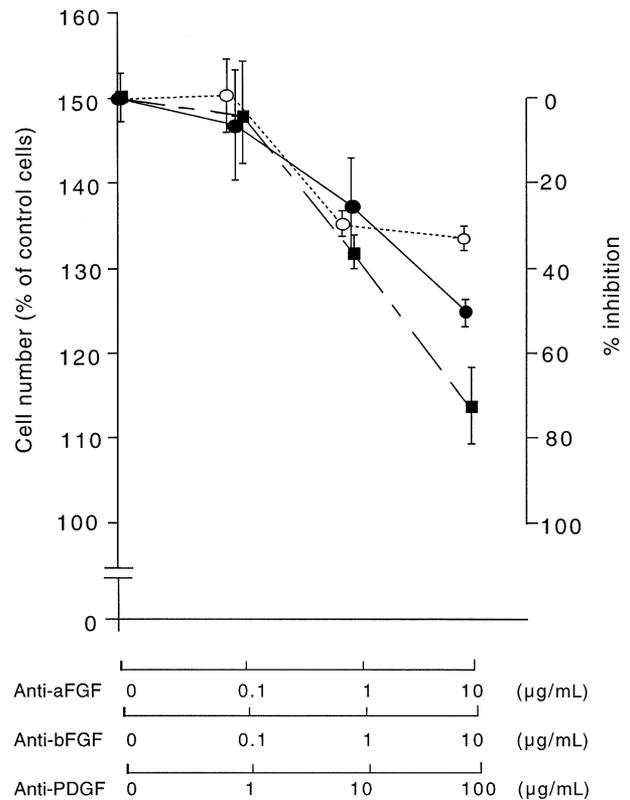
Figure 3. Neutralizing antibodies for acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) attenuate the effect of retinal glial cell (RGC) conditioned medium (CM). Cells were grown for 3 days in Dulbecco's modified Eagle's medium with 1% fetal bovine serum and RGC-CM (50% of the total volume), in the presence of 0.1, 1, or 10 mg/mL of antibody for aFGF (○), bFGF (●) or 1, 10, or 100 mg/mL antibody for PDGF (■). The cell number was determined after 3 days. The data are shown as a percentage of control cells (Y-axis left). The inhibition rate (Y-axis, right) of the effect of the RGC-CM is based on the number of control cells cultured without RGC-CM (negative control) and those cultured with 50% RGC-CM, or with control IgG (positive control). All antibodies showed a dose-dependent blocking effect. The maximum inhibition rate observed here was approximately 30% for aFGF, 50% for bFGF, and 70% for PDGF, respectively. The assay was triplicated, and the data indicates mean ± SD. These are representative data from three independent experiments.

Discussion

It has been reported that bovine retinal microvascular pericyte growth is stimulated by aFGF, bFGF, and the PDGF-B chain, but not by the PDGF-A chain, TGFβ2, or VEGF.[10] In addition, chick embryo RGCs have been reported to produce aFGF and bFGF.[11] The finding of the present study that
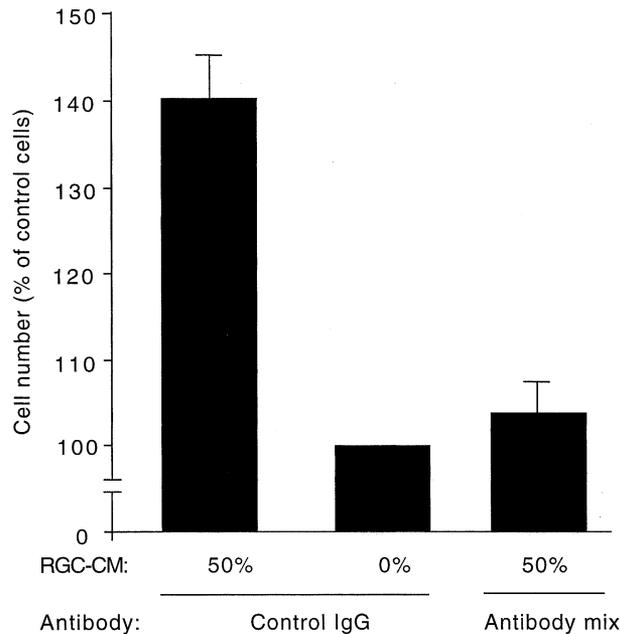
Figure 4. The effect of retinal glial cell (RGC) conditioned medium (CM) was completely blocked by inhibiting acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF). Cells were grown in Dulbecco's modified Eagle's medium with 1% fetal bovine serum with 50% RGC-CM or control CM. A mixture of antibodies (antibody mix) of 10 μg/mL of neutralizing anti-aFGF,10 μg/mL of anti-bFGF, and 100 μg/mL of anti-PDGF (100 μg/mL), or control IgG (120 μg/mL) were added as indicated. The cell number was measured 3 days later and mean ± SD were obtained. The stimulating effect of RGC-CM was almost completely blocked (over 90% inhibition) by the mixture of the three antibodies for aFGF, bFGF, and PDGF. The assay was triplicated, and these are representative data from three independent experiments.

Figure 5. RNA for acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), and , transforming growth factor-β 2(TGFβ2) is expressed in cultured RGCs. RNA was isolated from the cultured retinal glial cells (RGCs), and reverse transcription polymerase chain reaction was performed to detect the RNA expression for aFGF, bFGF, and TGFβ2. Synthesized cDNA was analyzed on the gel and stained with ethidium bromide. The corresponding cDNA was detected on the 3% agarose gel. Estimated size for aFGF, bFGF, and TGFβ2 was 270, 269, and 261 base pairs (bp), respectively. M: 100-bp marker.

RGCs stimulate pericytes through aFGF, bFGF, and PDGF-B is consistent with these previous reports. TGF-β2 was also found to be expressed in RGCs by RT-PCR, as is consistent with the finding by Ikeda et al with human glial cells.[12] However TGFβ2 did not seem to have a stimulative effect on pericytes in the present in vitro study. Thus, TGFβ2 from RGCs may not have a significant effect on pericyte proliferation. In conclusion, RGC seems to modulate pericyte growth mainly through the aFGF, bFGF, and PDGF-B chains.

Of the factors tested in this study, PDGF-BB showed a significant stimulating effect on pericyte growth. This finding is in agreement with the observation that the lack of PDGF-B, in particular, was found to result in critical changes such as pericyte deficiencies and consequent formation of microaneurysm in the retina of PDGF-B-deficient mice.[13] The mRNA for the PDGF-B chain and that for PDGF receptor have been reported to be expressed in the retina close to the vessels in the neonatal rodent retina.[14] Also, the PDGF-B chain plays an important role in vascular maturation.[15] Thus, it seems that the PDGF-B chain plays an important role in pericyte homeostasis, and the present study has shown that retinal glial cells are one of the contributors.

The mechanisms of pericyte loss during the progression of diabetic retinopathy remain unclear. Oxidative stress, such as advanced glycation end products, is believed to cause pericyte loss in diabetic retinopathy.[16] The RGCs have been shown to be damaged even during the early stages of diabetic retinopathy.[17] Based on these findings, it is possible that RGC damage due to diabetic retinopathy comes first, and that pericyte loss is a subsequent event. However, this issue is not presently clear and will require further study.

In vascular eyes, retinal vessels develop via the guidance of astrocytes migrating from the optic nerve head to peripheral retina.[18] The strongest candidate for a modulator of this guidance is thought to be VEGF.[19] This observation seems somewhat inconsistent with our data that pericyte growth is modulated by RGCs, such as aFGF, bFGF, and PDGF-BB, but not by VEGF. There are two explanations for this. One is that astrocytes and Müller cells are not identical, although they are believed to have the similar functions in many respects.[19] The other is that VEGF may act on pericytes as an chemotactant

in vivo.[20] Thus, the role of VEGF on pericytes in vivo still requires further investigation.

Because pericyte loss is thought to be the initial event for retinal neovascularization, it is very critical to understand how pericyte growth is modulated in order to develop new treatment for diabetic retinopathy. Our data is the first to provide a concept of how retinal pericyte growth is regulated by RGCs, and also may aid in developing a new treatment to prevent one of the early changes of diabetic retinopathy, pericyte loss. We believe that future studies will find a new aspect of the interaction between these two types of cells, and will supply further information regarding the pathogenesis of diabetic retinopathy.

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