Role of Vitronectin Receptor-Type Integrins and Osteopontin in Ischemia-Induced Retinal Neovascularization

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Purpose: It has been reported that vitronectin receptor-type integrins mediate vascular cell proliferation and migration. In this study, we investigated the expression of vitronectin receptor-type integrins and osteopontin in ischemia-induced retinal neovascularization, and examined the role of osteopontin in angiogenesis as a ligand of vitronectin receptor-type integrins.

Methods: Retinal neovascularization was produced by exposing C57BL/6J mice to 75% oxygen from postnatal day (P) 7 to P12. Expression of vitronectin receptor-type integrins and osteopontin was assessed by Northern blot analysis, in situ hybridization, and immunofluorescence. The role of osteopontin in retinal angiogenesis was evaluated by tube formation assay using cultured bovine retinal microcapillary endothelial cells.

Results: In the murine model, integrin αv mRNA was increased from P14 with a 2.6-fold peak response observed on P19, when retinal neovascularization was remarkable. Indirect immunofluorescence for vitronectin receptor-type integrins revealed prominent expression of integrin $\alpha v \beta 3/\beta 5$ in the neovascular endothelial cells. Osteopontin mRNA was increased from P14, with a 2.0-fold peak response observed on P19. In situ hybridization demonstrated localization of osteopontin mRNA in neovascular tufts. Vascular endothelial growth factor-induced tube formation (8.3 ± 0.6 mm/field) was inhibited significantly by treatment with anti-osteopontin antibody (4.8 ± 0.7 mm/field, $P < .001$).

Conclusions: These data suggest that increased expression of both vitronectin receptor-type integrins and osteopontin in ischemic retina contribute to vascular endothelial cell proliferation and to retinal vascular formation by promoting interaction between endothelial cells and extracellular matrix, which leads to retinal neovascularization. **Jpn J Ophthalmol 2002;46:270–278** © 2002 Japanese Ophthalmological Society

Key Words: Integrin, neovascularization, osteopontin, retinal ischemia, vitronectin.

Introduction

Integrins are heterodimers whose binding specificity is generated by various combinations of over 15 α and 8 β subunits that are expressed on cell surfaces in over 20 different $\alpha\beta$ heterodimetric combinations.[1,2] Studies have shown that $\alpha v \beta 3$ is selectively displayed on actively proliferating vascular endothelial cells and is involved in angiogenic processes.[3,4] Antagonists to integrin specifically and potently inhibit hypoxia-, cytokine-, and tumor-mediated angiogenesis in several animal models.[5–9] Other studies have demonstrated that at least two angiogenic pathways exist that can be defined by their dependency on $\alpha v \beta 3$ and $\alpha v \beta 5$.[10] The selective display of $\alpha v \beta 3$, and $\alpha v \beta 5$ integrins on blood vessels in tissue ob-

tained from patients with active neovascular eye disease has been reported.[11]

Osteopontin is one of the ligands for vitronectin receptor-type integrin.[2,12–14] Osteopontin is a secreted glycosylated phosphoprotein that was originally isolated from bone,[15,16] and has since been demonstrated to be expressed in a variety of human tissues, including kidney, thyroid, gastrointestinal tract, breast, testis, endometrium, uterine decidual cells, and placenta.[17–20] There is evidence that osteopontin plays an important role in cell physiology at these sites, including cell adhesion and signaling, cell migration, regulation of intracellular calcium levels, and modulation of the immune response to infections and neoplasia.[12,21] Evidence is also emerging that osteopontin may play a role in angiogenesis and tissue remodeling.[4,22–26]

In the present study, we investigated the expression of vitronectin receptor-type integrins and osteopontin in a murine model of ischemia-induced retinal neovascularization, and examined the role of osteopontin in vascular endothelial growth factor (VEGF)-induced angiogenesis using bovine retinal microcapillary endothelial cells.

Materials and Methods

Materials

VEGF was purchased from Genzyme (Cambridge, MA, USA). Anti-human osteopontin antibody was obtained from Gibco BRL (Gaithersburg, MD, USA) and American Research Products (Belmont, MA, USA). Plasma-derived horse serum (PDHS) was obtained from Wheaton (Pipersville, PA, USA). ^{32}P-dATP was obtained from Amersham (Buckinghamshire, UK). Restriction enzymes were obtained from Takara Biomedicals (Tokyo). Other chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO, USA).

Animal Model

The study adhered to the ARVO Standards for the Use of Animals in Ophthalmic and Vision Research. To produce ischemia-induced retinal neovascularization, litters of 7-day-old (postnatal day [P] 7) C57BL/6J mice, along with their nursing mothers, were exposed to $75 \pm 2\%$ oxygen for 5 days and then returned to room air at age P12, as described.[27,28] Mice of the same age maintained in room air served as controls. Flat-mounted, fluorescein-conjugated dextran-perfused retinas were examined to assess the retinal vasculature.[28]

Tissue Preparation

Mice at different time points (P12 immediately after return to room air, P14, P17, P19, P23, and P26, n = 3 for each time point) were deeply anesthetized with pentobarbital sodium (100 mg/kg) and sacrificed by cardiac perfusion of 4% paraformaldehyde in phosphate-buffered saline (PBS). Eyes were enucleated and fixed in 4% paraformaldehyde at 4°C overnight, and embedded in paraffin. Serial 5-μm sections of the whole eyes were placed on microscope slides, and the slides were stored at 4°C. Several slides from each eye were also stained with hematoxylin-eosin to assess the retinal vasculature.

In Situ Hybridization

Human osteopontin cDNA[17] was obtained from the American Type Culture Collection (Rockville, MD, USA). A cDNA fragment corresponding to the 478 nucleotides encoding amino acids 84 to 243 was subcloned into the pBluescript II vector (Stratagene, La Jolla, CA, USA). Sense and anti-sense RNA probes were transcribed in vitro from the linearized plasmid by use of digoxigenin-labeled UTP and T7 or T3 RNA polymerase, according to the manufacturer's instructions (DIG RNA Labeling Kit SP6/T7, Boehringer Mannheim, Indianapolis, IN, USA). The size of the probes was reduced to 150 b on average by alkaline treatment.[29]

In situ hybridization was performed as previously described,[30] with minor modifications. All incubation steps were performed in a moist chamber, and all the buffers and glassware used for the detection of mRNA had been made RNase-free. Paraffin was removed from the sections by treatment with xylene. After this, they were rehydrated through a graded series of alcohol and rinsed with PBS. They were then immersed in 0.2 N HCl for 20 minutes and incubated in proteinase K (10 μg/mL) for 15 minutes at 37°C. After washing in PBS, the specimens were refixed in freshly prepared 4% paraformaldehyde in PBS for 5 minutes, and then immersed in glycine (2 mg/mL) in PBS, two times for 15 minutes each time. They were then immersed in 2 × standard sodium citrate (SSC)/50% deionized formamide and incubated with hybridization mixture for 18 hours at 45°C. The composition of the hybridization mixture was as follows: 1 mg/mL *Escherichia coli* tRNA, 20 mM Tris-HCl buffer (pH 8.0), 10 mM EDTA, 1 × Denhardt's solution, 300 mM NaCl, 50% deionized formamide, 10% dextran sulfate. The final concentration of the probe was 250 ng/mL. After hybridization, the specimens were washed as follows: with 2 ×

SSC/50% formamide for 1 hour at 45°C; and with 0.5 M NaCl/10 mM Tris-HCl buffer (pH 8.0), twice for 5 minutes; RNase (20 μg/mL) treatment for 30 minutes at 37°C; 2 × SSC/50% formamide for 1 hour at 45°C, 1 × SSC/50% formamide for 1 hour at 45°C; and 1 × SSC/50% formamide for 1 hour at room temperature.

Immunological detection of hybridized probes was performed as described previously[31] using a Nucleic Acid Detection Kit (Boehringer Mannheim). Alkaline phosphatase-conjugated F(ab) fragments of anti-digoxigenin antibody were applied overnight at 4°C. After development of precipitation of the color reagent, the specimens were fixed in 10% formalin for 20 minutes to preclude the precipitation of crystallized chromogenic substance. Finally, the slides were dehydrated through a graded series of alcohol, clarified with xylene, and coverslipped with xylene-based permanent mounting media for viewing.

Confocal Microscopy

For immunofluorescence analysis, all incubation steps were performed in a moist chamber, and rinses were performed by immersing the slides in a PBS bath. Paraffin was removed from the sections by treatment with xylene. Sections were rehydrated through a graded series of alcohol and rinsed with PBS. Each section was incubated for 20 minutes with blocking reagent (Dako, Glostrup, Denmark). The specimens were incubated overnight at 4°C with the anti-vitronectin receptor antibody, then washed for 30 minutes with PBS. The sections were incubated for 30 minutes with anti-rabbit IgG labeled with fluorescein isothiocyanate (Dako), washed for 30 minutes with PBS, and coverslipped with Vectashield (Vector, Burlingame, CA, USA) for viewing. For the negative control, normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. Other staining procedures were the same as described above.

All specimens were examined with a Zeiss scanning laser confocal microscope (LSM 410 invert Laser Scan Microscope, Zeiss, Oberkochen, Germany). Digitized images were captured by computer and stored on an optical disk for subsequent display. Photographic images were taken from the computer with a digital printer (Pictrography, Fuji Photo Film, Tokyo).

Cell Cultures

Primary cultures of bovine retinal endothelial cells (BREC) were isolated by homogenization and a series of filtration steps, as described previously.[32,33]

Primary BREC were grown on fibronectin-coated dishes (Iwaki Glass, Tokyo) containing Dulbecco's modified Eagle's medium (DMEM) with 5.5 mM glucose, 10% PDHS, 50 mg/L heparin, and 50 U/L endothelial cell growth factor (Boehringer Mannheim). The cells were cultured in 5% CO_2 at 37°C; medium was changed every 3 days. Endothelial cell homogeneity was confirmed by immunoreactivity with anti-factor VIII antibodies analyzed by confocal microscopy. After the cells reached confluence, the medium was changed every 3 days and cells from passages 3–10 were used for these experiments. Endothelial cell homogeneity and cellular characteristics, such as cell shape and growth rate were carefully observed until passage 15, and remained unchanged throughout the observation period.

Northern Blot Analysis

For the animal studies, total RNA was isolated from retinas of mice at different time points (10 retinas from 5 mice at each time point: P12 immediately after return to room air, P14, P17, P19, P23, and P26) using guanidium thiocyanate; for culture studies, total RNA was isolated from individual tissue culture plates. Northern blot analysis was performed on 15 μg total RNA after 1% agarose-2M formaldehyde gel electrophoresis and subsequent capillary transfer to Biodyne nylon membranes (Pall BioSupport, East Hills, NY, USA) and ultraviolet crosslinking using a FUNA-UV-LINKER (FS-1500, Funakoshi, Tokyo). Radioactive probes were generated using Amersham Megaprime labeling kits (Amersham) and ^{32}P-dATP. Blots were pre-hybridized, hybridized and washed in 0.5 × SSC, 5% SDS at 65°C with four changes over 1 hour in a rotating hybridization oven (Taitec, Koshigaya). All signals were analyzed using a densitometer (BAS-2000II, Fuji Photo Film) and lane loading differences were normalized using the 36B4 cDNA probe.[34]

Human osteopontin cDNA[17] was obtained from the American Type Culture Collection (Rockville, MD, USA). For human integrin αv cDNA, a standard polymerase chain reaction (PCR) was performed using CTTCAACCTAGACGTGGACAGT (sense primer) and TTGAAATCTCCGACAGCCACAG (anti-sense primer),[35] with cDNA from human umbilical vein endothelial cells as PCR templates. The PCR products were subcloned into the pBluescript II vector (Stratagene). The sequences of the subcloned DNA were confirmed by nucleotide sequencing, and we used these clones as human integrin αv probes.

Tube Formation Assay

An 8:1:1 volume of Vitrogen 100 (Celtrix, Palo Alto, CA, USA), 0.2 N NaOH and 200 mM HEPES, and 10 × RPMI medium (Gibco BRL) was made to 400 μL and added to 24-well plates. After polymerization of the gels, 1.0×10^5 BREC were seeded and incubated with DMEM containing 20% PDHS for 24 hours at 37°C, after which the medium was removed and additional collagen gel was placed on the cell layer. Anti-osteopontin antibody (10 μg/mL) or vehicle was added to the medium, and stimulated with VEGF (25 ng/mL). Five days later, five different fields (1.28 mm × 1.28 mm) were chosen and total tube-like structures were measured using NIH Image (by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Results are expressed as mean ± standard deviation, unless otherwise indicated. Statistical analysis employed Student's t-test. A P-value of $<.05$ was considered statistically significant.

Results

Integrin αv mRNA Levels in Hypoxic Retina

Similar to results of previous studies,[27,28] histologic examination of hematoxylin-eosin stained sections showed neovascular tufts, particularly in the midperiphery, extending above the internal limiting membrane into the vitreous after 5 days of hypoxia. These neovascular tufts were most prominent at P17–19; after P23 the neovascularization regressed, and the vascular pattern normalized by P26. In this model, vitronectin receptor-type integrins have been reported to play a critical role in retinal neovascularization.[8,9] Accordingly, we performed Northern blot analysis to investigate mRNA expression of integrin αv in retinal neovascularization. Figure 1 shows a prominent increase in integrin αv mRNA levels from P17 to P19. RNA from age-matched control animals raised in room air demonstrated a comparatively constant and low level of integrin αv mRNA. The fold increase in integrin αv mRNA at each time point, compared with age-matched controls after normalization to the 36B4 signal in each lane, showed a maximal 2.6-fold increase on P19 compared with normal age-matched controls.

Localization of Vitronectin Receptor-Type Integrins in Hypoxic Retina

To determine the location of the increased integrin αv expression during the development of neovascularization, indirect immunofluorescence for vitronectin receptor-type integrins was performed with
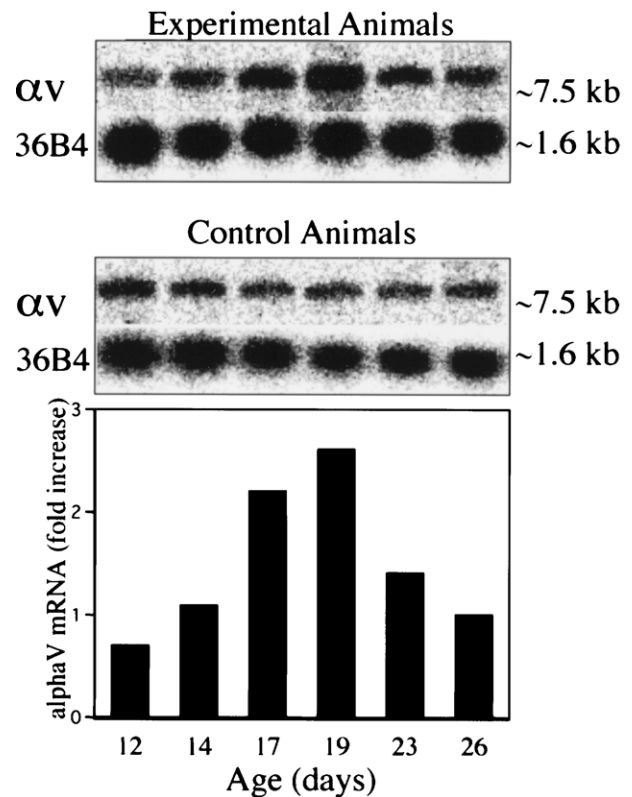

Figure 1. Integrin αv mRNA expression during hypoxia and the development of neovascularization in a mouse model of ischemia-induced retinal neovascularization. Results of the Northern blot analysis of total RNA (15 μg) isolated from animals after various durations of hypoxia and from age-matched normal controls. Northern blots and control 36B4 (top) and quantification (bottom). For these calculations, the amount of integrin αv mRNA at each time point was first normalized to its own 36B4 signal. The fold increase over the normalized value for corresponding age-matched normal controls was then calculated. A maximal 2.6-fold increase of integrin αv mRNA was observed on postnatal day 19. Similar data were obtained from another Northern blot analysis (data not shown).

confocal microscopy. Integrin αvβ3/β5 immunoreactivity was observed in the vascular cells in retinas from both hypoxic P19 mice (Figure 2B) and normal control P19 mice (Figure 2A). Low levels of integrin αvβ3/β5 protein were also detected in the ganglion cell layer and inner and outer nuclear layers. No integrin αvβ3/β5 immunoreactivity was seen in the negative control. Analysis of the pattern of integrin αvβ3/β5 protein expression demonstrated that, in hypoxic retina, the immunoreactivity of integrin αvβ3/β5 was increased in both intensity and in number of vessels involved near the avascular area, particularly at the neovascular tufts (Figure 2C). Simi-
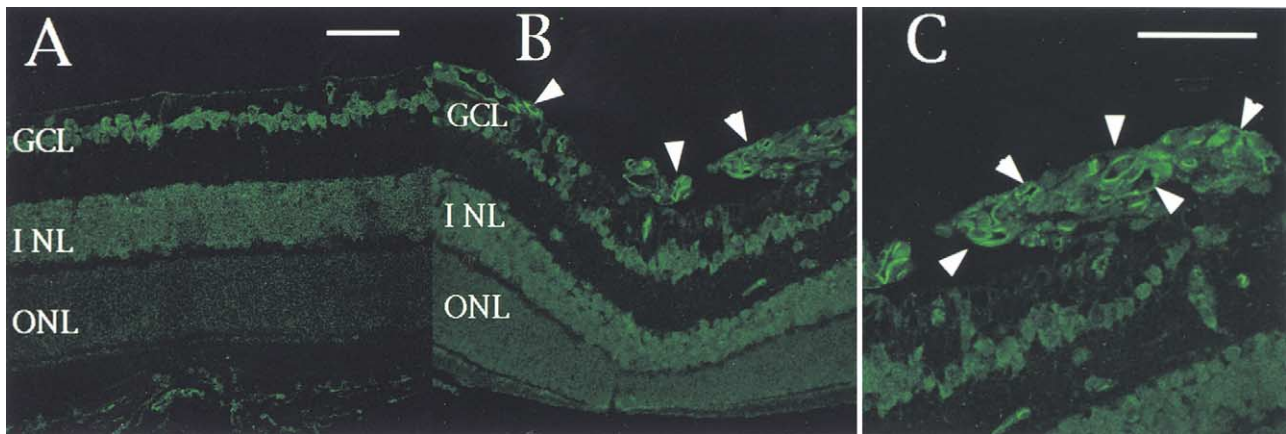
Figure 2. Localization of integrin αvβ3/β5 expression in hypoxic retina. Integrin αvβ3/β5 immunoreactivity was observed in the vascular cells in retinas from both hypoxic postnatal day 19 (P19) mice (**B**, arrowheads) and normal control P19 mice (**A**). Integrin αvβ3/β5 protein was also detected mildly in the ganglion cell layer and in the inner and outer nuclear layers. No integrin αvβ3/β5 immunoreactivity was seen in the negative controls. Analysis of the pattern of integrin αvβ3/β5 protein expression demonstrated that, in hypoxic retina, the immunoreactivity of integrin αvβ3/β5 was increased in both intensity and in number of vessels involved near the avascular area, particularly at the neovascular tufts (**C**, arrowheads). (**A**) and (**B**) are at the same magnification. Bar = 50 μm in (**A**) and (**C**).

larly, at other time points, integrin αvβ3/β5 was expressed prominently in vascular cells near the avascular area, while staining of other retinal layers was similar to that seen at P19.

Osteopontin mRNA Levels in Hypoxic Retina

We investigated mRNA expression of osteopontin in retinal neovascularization. Figure 3 shows an increase in osteopontin mRNA levels from P17 to P19 compared to age-matched control animals raised in room air. The fold increase in osteopontin mRNA at each time point, compared with age-matched controls after normalization to the 36B4 signal in each lane, showed a maximal 2.0-fold increase on P19. In contrast, vitronectin mRNA levels increased gradually from P12 to P26 in both hypoxic and control retinas. The fold increase in vitronectin mRNA at each time point, compared with age-matched controls after normalization to the 36B4 signal in each lane, were almost identical.

Localization of Osteopontin mRNA in Hypoxic Retina

To determine the location of the upregulated osteopontin mRNA expression during the development of neovascularization, in situ hybridization was performed. Osteopontin mRNA was observed in the vascular cells of retinas from hypoxic P19 mice (Figure 4B), but also, albeit more weakly, in the vascular cells of retinas from normal control P19 mice (Figure 4A). No strong localization of osteopontin mRNA was de-

tected in other retinal cells. No signal was seen in the negative controls hybridized with the sense probe (Figure 4C). The pattern of osteopontin mRNA localization demonstrated that, in hypoxic retina, the strongest signal was detected in vessels near the avascular area, particularly at the neovascular tufts (Figure 4D). Furthermore, using cultured BREC, we performed Northern blot analysis to see if retinal vascular cells express osteopontin and vitronectin mRNA; as shown in Figure 5, results suggested that osteopontin is predominantly expressed in BREC.

Anti-Osteopontin Antibody Inhibits VEGF-Induced Tube Formation in BREC

Because VEGF is expressed prominently in our mouse model,[27] and plays a central role in retinal neovascularization,[36,37] we evaluated the effects of anti-osteopontin antibody on VEGF-induced tube formation in BREC. In three independent experiments, VEGF induced tube formation at 8.3 ± 0.6 mm/field, while treatment with anti-osteopontin antibody inhibited the VEGF-induced tube formation in BREC by 4.8 ± 0.7 mm/field in comparison to VEGF stimulation alone ($P < .001$). Nonspecific IgG had no effect on VEGF-induced tube formation in BREC.

Discussion

Binding specificity of integrins is generated by various combinations of their subunits, and emerging
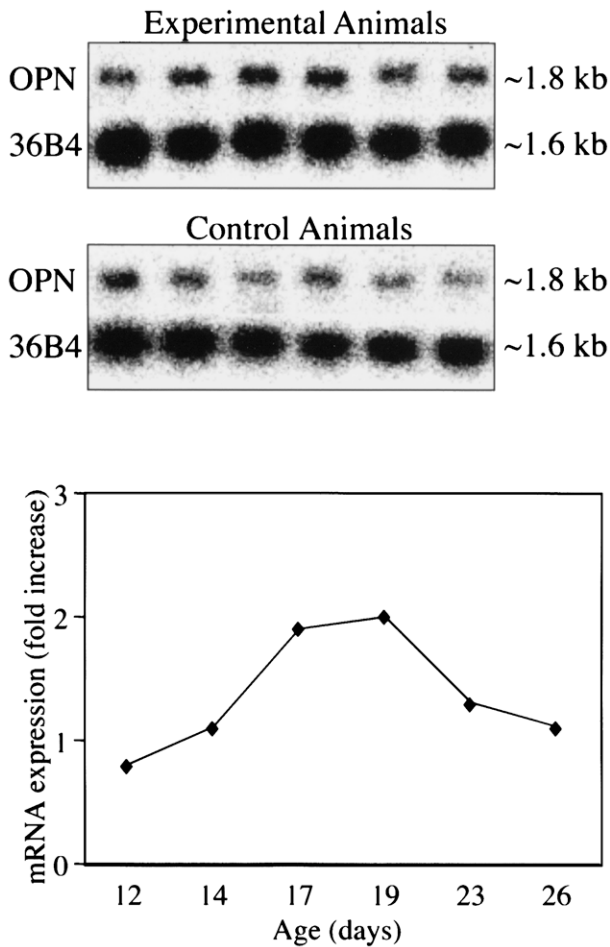
Figure 3. Osteopontin (OPN) mRNA expression during hypoxia and the development of neovascularization. Results of the Northern blot analysis of total RNA (15 µg) isolated from animals after various durations of hypoxia and from age-matched normal controls. Northern blots and control 36B4 (top) and quantification (bottom). For these calculations, the amount of osteopontin mRNA at each time point was first normalized to its own 36B4 signal. The fold increase over the normalized value for the corresponding age-matched normal control was then calculated. A maximal 2.0-fold increase of osteopontin mRNA was observed on postnatal day 19.
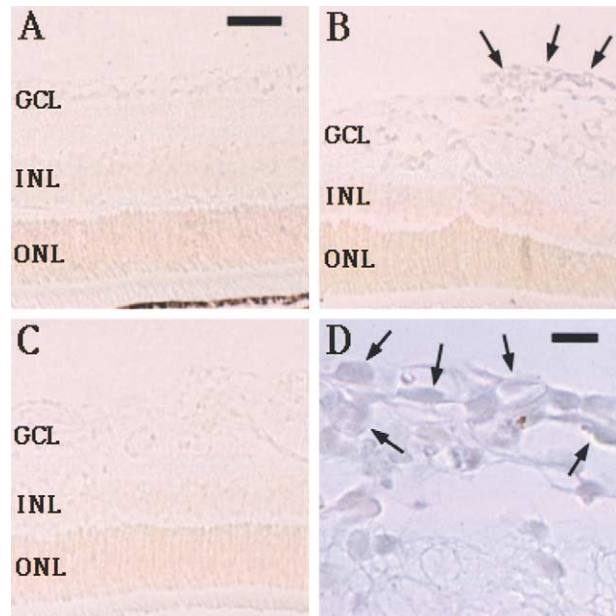

Figure 4. Localization of osteopontin mRNA expression in hypoxic retina. Osteopontin mRNA were observed in vascular cells of retinas from hypoxic postnatal 19 (P19) mice (**B**, arrows) and weakly in retinas from normal control P19 mice (**A**). No strong localization of osteopontin mRNA was detected in other retinal cells. No signal was seen in the negative control hybridized with sense probe (**C**). The pattern of osteopontin mRNA localization demonstrated that, in hypoxic retina, strong signals were detected in vessels near the avascular area, particularly at the neovascular tufts (**D**, arrows). (**A**), (**B**), and (**C**) are at the same magnification. Bar = 50 µm in (**A**) and 10 µm in (**D**).

evidence suggests that individual integrins mediate distinct functions and elicit distinct signaling pathways. For example, members of the α5 and α2 integrin family are reported to be receptors primarily for fibronectin and collagen,[2,38] and the αvβ1, αvβ3, and αvβ5 integrins are receptors mainly for vitronectin and osteopontin.[12–14] It has been reported that the dominant role of some β1 integrin family members is the anchoring of cells to the matrix,[39] but αvβ3 and αvβ5 integrins play essential roles in cell migration[39,40] and in angiogenesis.[6,10,11] It is also reported that migration of smooth muscle cells in response to osteopontin depends on integrin αvβ3, while adhesion to osteopontin is supported by the αvβ1, αvβ3, and αvβ5 integrins.[21] NF-kappaB has been suggested as an important signaling molecule in αvβ3 integrin-mediated endothelial cell survival.[26] VEGF was reported to induce both a ligand, osteopontin, and its receptor, integrin αvβ3, in dermal microvascular endothelial cells to enhance endothelial cell migration, suggesting a role for VEGF as a regulator of angiogenic integrin and its ligand system.[4] Our previous study revealed that hypoxia upregulates the integrin expression via VEGF induction in bovine retinal capillary endothelial cells.[41]

In the present study, we have demonstrated that the expression of vitronectin receptor-type integrins and its ligand, osteopontin, are increased in hypoxia-induced retinal neovascularization, and that osteopontin serves as an essential extracellular matrix to regulate the angiogenic process in retinal capillary endothelial cells.
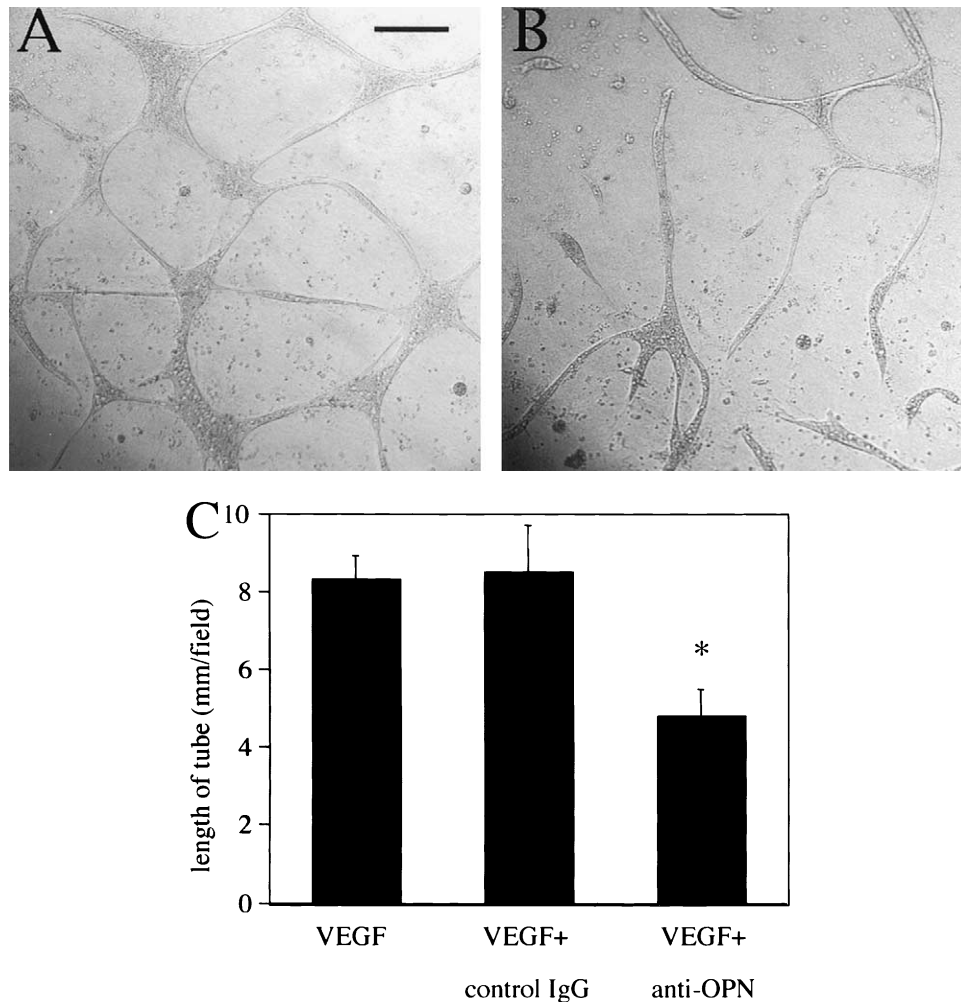
Figure 5. Tube formation by bovine retinal endothelial cells (BREC). BREC were seeded in three-dimensional collagen gel and incubated with the medium containing vascular endothelial growth factor (VEGF) or VEGF and antibodies for 5 days. Total length of tube formation in each well was measured and length/one field were compared. Representative phase-contrast micrographs of tube formation in 3 experiments; treated with VEGF (**A**), treated with VEGF and anti-osteopontin (OPN) antibody (**B**), and summarized results are shown (**C**). (**A**) and (**B**) are at the same magnification. Bar = 200 μm in (**A**).

In the ischemia-induced retinal neovascularization, vitronectin receptor-type integrins were reported to have a critical role in retinal neovascularization.[8,9] Accordingly, we investigated mRNA expression of integrin αv in the ischemic retina, and found a marked increase in integrin αv mRNA levels from P17 to P19, when the retinal neovascularization was most prominent.[27,28] We also performed indirect immunofluorescence to localize vitronectin receptor-type integrin expression in ischemic retina, and observed increases of integrin αvβ3/β5 expression in both retinal and intravitreally growing vascular cells. This increase was more evident in neovascular cells growing into the vitreous, and the most marked expression was observed at the neovascular tufts adjacent to areas of retinal nonperfusion. These data suggest a possibility that angiogenic vascular cells in ischemic conditions express more vitronectin receptor-type integrins than do static and normoxic vascular cells.

It has been reported that the main ligands of vitronectin receptor-type integrins include osteopontin.[2] Accordingly, we investigated mRNA expression of osteopontin in ischemia-induced retinal neovascularization, and found an increase in osteopontin mRNA levels with a maximal 2.0-fold increase on P19 compared with normal age-matched controls. In situ hybridization also revealed increases of osteopontin expression in both retinal and intravitreally growing vascular cells. As integrin αvβ3/β5, this increase was more evident in neovascular cells grow-

ing into the vitreous, and the most marked expression was observed at the neovascular tufts adjacent to areas of retinal nonperfusion.

In the model, neovascularization is developing from P13 and is the most prominent during P17 to P21.[28] Both the ligand and the matrix receptor are gradually co-upregulated along with the development of neovascular vessels and peak when the neovascularization is most prominent. These data suggest that the co-localized induction of osteopontin and integrin αv in ischemic retinal neovascular cells might contribute to retinal vascular cell migration and angiogenesis in ischemia-induced retinal neovascularization.

In the mouse models, the induction of VEGF is closely associated with the development of neovascularization[27]; inhibition of VEGF results in suppression of retinal neovascularization[36,37]; the VEGF induction is concentrated just posterior to the neovascular tufts.[42] Further, VEGF mRNA level peaked at P12 and upregulated during the time period of neovascular development,[42] which is just prior to the osteopontin and integrin increase in the present study. It is also reported that VEGF induces both a ligand, osteopontin, and its receptor, integrin αvβ3, in dermal microvascular endothelial cells to enhance endothelial cell migration.[4] These data might suggest that the induction of vitronectin receptor-type integrins and osteopontin in ischemic retina could be mediated predominantly through hypoxic VEGF induction.

Because VEGF is expressed prominently in the mouse model,[27] and plays a central role in retinal neovascularization,[36,37] we evaluated the effects of anti-osteopontin antibody on VEGF-induced tube formation in BREC to assess the role of osteopontin in angiogenesis. In our experiments, treatment with anti-osteopontin antibody markedly inhibited the VEGF-induced tube formation. These data suggest that osteopontin is essential in VEGF-stimulated angiogenesis in the in vitro model. Further studies including an in vivo analysis are necessary to elucidate its substantial role in ischemia-induced retinal neovascularization in ischemic retinal diseases. Osteopontin contains the adhesive motif, arginine-glycine-aspartate (RGD),[12] which is thought to be recognized by integrin αvβ3,[43,44] but the αv-containing receptors may also interact with other regions of osteopontin.[45] It is also reported that migration in response to osteopontin depends on integrin αvβ3, while integrins αvβ1, αvβ3, and αvβ5 function as osteopontin adhesive receptors.[21] In our tube formation assay using BREC, the interaction between integrin αvβ3 and the RGD regions of osteopontin could play a critical role, but further investigation is

needed to clearly define the mechanism of integrins-osteopontin interaction in angiogenesis.

In conclusion, these data suggest that increased co-upregulation of vitronectin receptor-type integrins and osteopontin in ischemic retina contributes to vascular endothelial cell proliferation and retinal vascular formation by promoting interaction between endothelial cells and extracellular matrix,[3,46] and that these specific increases in the αv integrin family and osteopontin potentiate neovascularization in ischemic retina.

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