Osteoprotegerin Is a New Regulator of Inflammation and Angiogenesis in Proliferative Diabetic Retinopathy

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PURPOSE. Osteoprotegerin (OPG) is a novel regulator of endothelial cell function, angiogenesis, and vasculogenesis. We correlated expression levels of OPG with those of the angiogenic and inflammatory factors vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1/CCL2) in proliferative diabetic retinopathy (PDR). We also examined expression of OPG in retinas from diabetic rats and diabetic patients and measured production of OPG by human retinal microvascular endothelial cells (HRMEC) and investigated its angiogenic activity.

METHODS. Vitreous samples from 47 PDR and 28 nondiabetic patients, epiretinal membranes from 14 patients with PDR, human retinas (10 from diabetic patients and 10 from nondiabetic subjects), and rat retinas and HRMEC were studied by using enzyme-linked immunosorbent assay, immunohistochemistry, immunofluorescence, Western blot analysis, and RT-PCR. In vitro and in vivo angiogenesis assays were performed.

RESULTS. We showed a significant increase in the expression of OPG, VEGF, and MCP-1/CCL2 in a comparison between vitreous samples from PDR patients and those from nondiabetic controls. Significant positive correlations were found between levels of OPG and levels of VEGF and MCP-1/CCL2. In epiretinal membranes, OPG was expressed in vascular endothelial cells and stromal cells. Significant increases of OPG mRNA and protein were detected in the retinas from diabetic patients. The proinflammatory cytokines TNF-α and IL-1β, but not VEGF, MCP-1/CCL2 or thrombin, induced upregulation of OPG in HRMEC. Osteoprotegerin induced ERK1/2 and Akt phosphorylation in HRMEC and stimulated their migration. Osteoprotegerin potentiated the angiogenic effect of VEGF in the in vivo protein gelatin plug assay.

CONCLUSIONS. These results suggest that OPG is involved in PDR angiogenesis.

Keywords: angiogenesis, monocyte chemoattractant protein-1, osteoprotegerin, proliferative diabetic retinopathy, vascular endothelial growth factor

Ischemia-induced retinal angiogenesis and vasculogenesis are hallmark features of proliferative diabetic retinopathy (PDR) and are critical steps for the development and progression of PDR.1-4 Pathologic growth of new blood vessels and expansion of extracellular matrix in association with the outgrowth of fibrovascular epiretinal membranes at the vitreoretinal interface often leads to catastrophic loss of vision due to vitreous hemorrhage and/or traction retinal detachment. The dynamic balance between proangiogenic and antiangiogenic factors is thought to regulate angiogenesis.5 Vascular endothelial growth factor (VEGF) is the major angiogenic factor in PDR that promotes neovascularization and vascular leakage.6 In several studies, the overexpression of proinflammatory and proangiogenic molecules was demonstrated in the ocular microenvironment of patients with PDR,7-12 suggesting that persistent inflammation and neovascularization are critical for PDR initiation and progression.

Our understanding of the precise molecular mechanisms responsible for the regulation of retinal angiogenesis and vasculogenesis in patients with PDR is incomplete and molecular regulation of these processes is the subject of many studies. A potential novel regulator of endothelial cell function, angiogenesis, and vasculogenesis is osteoprotegerin (OPG), a member of the TNF receptor superfamily. Osteoprotegerin lacks a transmembrane domain and, unlike most members of this family, is secreted into the extracellular space.13-14 Osteoprotegerin was initially identified for its role in regulating bone metabolism by mediating paracrine signaling between osteoblasts and osteoclasts.15-16 In addition to its role in bone metabolism, OPG has recently been found to have additional
roles in regulating endothelial cell function in tumor angiogenesis and vascular disease.\textsuperscript{13,14} In addition, OPG may be involved in development of vascular endothelial cell dysfunction and micro- and macrovascular complications in diabetes.\textsuperscript{17,18} The expression of OPG in PDR has not been reported so far. Given the key roles of OPG in regulating endothelial cell function and promotion of angiogenesis, we investigated the hypothesis that OPG may be involved in PDR pathogenesis.

**MATERIALS AND METHODS**

**Vitreous Fluid Samples and Epiretinal Membranes Specimens**

Undiluted vitreous fluid samples were obtained from 47 PDR patients during pars plana vitrectomy as described previously.\textsuperscript{7–12} The indications for vitrectomy were tractional retinal detachment and/or nonclearing vitreous hemorrhage. The control group consisted of 28 patients who had undergone vitrectomy for the treatment of rhegmatogenous retinal detachment with no proliferative vitreoretinopathy (PVR). Controls were free from systemic disease. Epiretinal fibrovascular membranes were obtained from 14 PDR patients during pars plana vitrectomy for the repair of tractional retinal detachment. Control epiretinal membranes were obtained from 10 nondiabetic patients undergoing vitrectomy for PVR. Membranes were fixed for 2 hours in 10\% formalin solution and embedded in paraffin.

The study was conducted according to the tenets of the Declaration of Helsinki. All patients were candidates for vitrectomy as a surgical procedure. All patients signed a preoperative informed written consent and approved the use of the excised epiretinal membranes and vitreous fluid for clinical research. The study design and protocol were approved by the Research Centre and Institutional Review Board of the College of Medicine, King Saud University.

**Human Postmortem Eyes**

Human postmortem eyes were obtained from 10 diabetic individuals with mild nonproliferative diabetic retinopathy and 10 nondiabetic (control group) donors matched by age. After enucleation, one eye from each donor was snap-frozen in liquid nitrogen at \(-80\)\(^\circ\)C and stored until assayed for mRNA or protein analyses. Neuroretina and retinal pigment epithelium (RPE) were quickly harvested. Vitreous and neuroretinas were removed, and the RPE layer was carefully peeled from Bruch’s membrane, using forceps (Dumont no. 5; Sigma, Madrid, Spain) under a dissecting microscope (model SZ61; Olympus, Barcelona, Spain). The other eye was fixed in 4\% paraformaldehyde and embedded in paraffin.

All ocular tissues were used in accordance with applicable laws and with tenets of the Declaration of Helsinki for research involving human tissue. In addition, this study was approved by the Ethics Committee, Institut de Recerca Hospital Universitari Vall d’Hebron.

**Rat Streptozotocin-Induced Diabetes Model**

All procedures with animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the College of Pharmacy, King Saud University. Diabetes was induced in adult male Sprague-Dawley rats, by intraperitoneal injection of strepto-
were obtained from age-matched nondiabetic control rats. SDS-PAGE. Equal volumes (15 μL) of vitreous fluid samples from patients with PDR (n = 16) and from nondiabetic patients with rhegmatogenous RD (n = 8) were analyzed using PAGE, and the presence of OPG was detected by using Western blot analysis. A representative set of samples (three RD samples and three PDR samples) is shown (left panel). The intensity of the protein band around 50 kDa was determined in all samples (right panel). *P < 0.05 (Mann-Whitney U test).

**Enzyme-Linked Immunosorbent Assays for Vitreous Samples**

Enzyme-linked immunosorbent assay (ELISA) kits for human osteoprotegerin (catalog no. DY805), MCP-1/CCL2 (catalog no. DCP00), and VEGF (catalog no. SVE00) were purchased from R&D Systems, Minneapolis, MN, USA.

**Western Blot Analysis for OPG Expression in Human Vitreous Fluid Samples and Rat Retinas**

Retinas from diabetic and control rats were homogenized in Western blot lysis buffer as described previously, and homogenates were subjected to sodium dodecyl sulfate (SDS)-PAGE. Equal volumes (15 μL) of vitreous samples were boiled (10 min) in Laemmli’s sample buffer (1:1 v/v) under reducing conditions and analyzed as described previously.

Blots were incubated overnight with anti-OPG antibody (1:1000 dilution; clone ab73400; Abcam) as described. To identify the phenotype of cells expressing OPG, sequential double immunohistochemistry was performed as described.

**Immunohistochemical Staining of Epiretinal Membranes**

For CD31, α-smooth muscle actin (α-SMA), and OPG detection, antigen retrieval (10-minute incubation) was performed using citrate based buffer (pH 5.9 to 6.1; Bond epitope retrieval solution 1; Leica Biosystems, Buffalo Grove, IL, USA). For CD45 detection, antigen retrieval (20-minute incubation) was performed using Tris/EDTA buffer (pH 9) (Bond epitope retrieval solution 2; Leica). Subsequently, the sections were incubated (60 minutes) with mouse monoclonal anti-CD31 antibody (clone JC70A; Dako, Glostrup, Denmark), mouse monoclonal anti-CD45 antibody (clones 2B11 and PD7/26; Dako), mouse monoclonal anti-α-SMA antibody (clone 1A4; Dako), and rabbit polyclonal anti-OPG antibody (1:50 dilution; clone ab9986; Abcam) as described. To identify the phenotype of cells expressing OPG, sequential double immunohistochemistry was performed as described.

**Immunofluorescent Detection of OPG in Retinas From Diabetic Patients**

Retinal sections (7-μm thickness) were pretreated as described, blocked (2% bovine serum albumin in 0.05% Tween in phosphate-buffered saline for 1 hour), and incubated overnight with primary antibody against OPG (1:500 dilution; clone ab73400; Abcam). Colocalization studies were performed using anti-glial fibrillary acidic protein (GFAP) (1:1000 dilution; clone ab10062; Abcam), anti-Iba1 (1:200 dilution; clone ab5076; Abcam), and anti-collagen IV (1:50 dilution; clone ab6511; Abcam). After sections were washed, they were incubated with Alexa Fluor 488 or 594 secondary antibodies (Molecular Probes, Invitrogen, Madrid, Spain) at room temperature for 1 hour. Slides were cover-slipped with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) for visualization of cell nuclei (Vector Laboratories, Pyles, Sant Cugat del Vallés, Spain). Images were acquired with a confocal laser scanning microscope (model FV1000; Olympus).

**Analysis of OPG Expression in Retinas From Diabetic Patients**

Human neuroretina and RPE were harvested under the microscope. For Western blot analysis, neuroretina and RPE samples were extracted with radioimmunoprecipitation assay buffer and homogenized by sonication. The protein concentration was determined using a bicinchoninic acid protein assay (Bio-Rad Laboratories, Madrid, Spain). Protein extracts (20 μg) were resolved by 10% SDS-PAGE and transferred to ECL nitrocellulose membranes (Hybond; Amersham Pharmacia Biotech, Little Chalfont, UK). Membranes were first incubated with anti-OPG (1:1000 dilution; clone ab73400; Abcam) and afterward with peroxidase-conjugated secondary antibody (Bio-Rad Laboratories). Proteins were visualized using the enhanced chemiluminescence detection system (Supersignal CL-horseradish peroxidase substrate system; Thermo Scientific, Rockford, IL, USA).

**FIGURE 2.** Determination of OPG levels in vitreous fluid. Equal volumes (15 μL) of vitreous fluid samples from patients with PDR (n = 16) and from nondiabetic patients with rhegmatogenous RD (n = 8) were analyzed using PAGE, and the presence of OPG was detected by using Western blot analysis. A representative set of samples (three RD samples and three PDR samples) is shown (left panel). The intensity of the protein band around 50 kDa was determined in all samples (right panel). *P < 0.05 (Mann-Whitney U test).
Total RNA was extracted from isolated retinal tissues by using Trizol reagent (Invitrogen). Concentrations of RNA were determined, and reverse transcription was performed using 1 μg of total RNA, using random hexanucleotide priming and reagents provided by Applied Biosystems, Madrid, Spain. The power SYBR Green PCR Master Mix (Applied Biosystems) was used for real-time PCR, and reactions were conducted at 95°C for 10 min, for 50 cycles of 15 seconds each at 95°C, and for 1 minute at 60°C, using the Prism 7000 unit (Applied Biosystems). Each sample was assayed in duplicate, and control negative samples were included in each experiment. Specific primer pairs corresponding to human OPG (forward primer 5’-TGGCACCAAAGTAAACGCAGAG-3’ and reverse primer 5’-CTCGAAGGTGAGGTTAGCATGTC-3’) and human β-actin (as endogenous control: forward primer 5’-TGGAGAAAATCTGGCACCAC-3’ and reverse primer 5’-GAGGCGTACAGGGATAGCAC-3’) were used.

**Induction of Human Retinal Microvascular Endothelial Cells**

Human retinal microvascular endothelial cells (HRMEC; Cell Systems, Kirkland, WA, USA) were cultured as described.10 Confluent monolayers were stimulated with VEGF (Biolegend, San Diego, CA, USA), IL-1β (Peprotech, Rocky Hill, NJ, USA), TNF-α (Peprotech), MCP-1/CCL2 (Peprotech) and thrombin (Sigma). In addition, 25 mM of mannose (osmotic control) or 25 mM of glucose was added to the stimulation medium to evaluate.

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**FIGURE 3.** Osteoprotegerin is expressed in epiretinal membranes from patients with PDR. Expression of OPG in PDR epiretinal membranes was investigated by immunohistochemistry analysis using a specific anti-OPG antibody. Specificity was checked by use of an irrelevant polyclonal antibody (A) (negative control). Microvessels, infiltrating leukocytes, and myofibroblasts were stained using anti-CD31 (B), anti-CD45 (C), and anti-α-smooth muscle actin (D) antibodies, respectively. Immunoreactivity for OPG was observed in the vascular endothelium (arrows) (E) and in stromal spindle-shaped cells (arrowbeads) (F). Double-immunohistochemistry analysis was performed using CD45 (brown) and OPG (red), and co-staining was detected in stromal cells (arrows) (G) (original magnification ×40).
the effect of hyperglycemia. All inductions were performed in duplicate. After 72 hours, cell supernatants were harvested, centrifuged to remove cell debris and stored at \(-20^\circ C\).

**Signal Transduction Assays for Phosphorylated Akt and Extracellular Signal-Regulated Kinase (ERK)1/2**

Signal transduction experiments with HRMEC were performed as described previously.\(^{10}\)

**Chemotaxis Assay**

The chemotactic activity of recombinant human OPG (TNFRSF11B; R&D Systems) for HRMEC was evaluated using cell invasion/migration plates and the RTCA-DPx CELLigence instrument (Acca Biosciences, San Diego, CA, USA). This system records changes of electrical impedance, which is expressed as cell index (CI) over a prolonged time course. First, 160 \(\mu\)L of stimulus (control medium, OPG, or VEGF) and 50 \(\mu\)L of EBM-2 with 0.4% fetal bovine serum (FBS; control medium) was added to the lower and upper chambers, respectively. Subsequently, the CIM16-plate was equilibrated (1 hour, \(37^\circ C\)). Afterward, HRMECs (40,000 cells per well; 100 \(\mu\)L/well) were seeded in the upper chamber in EBM-2 medium containing 0.4% FBS and allowed to settle onto the membrane (30 minutes, \(20^\circ C\)). After transfer to a CO\(_2\) incubator (\(37^\circ C\)), the impedance value was monitored every minute during 15 hours and expressed as CI. Cells migrating across the membrane cause an increase in the CI. Each experiment was performed in duplicate.

**In Vivo Gelatin Protein Plug Assay**

Cooled growth factor-reduced protein gelatin (9 to 11 mg of protein/mL; Matrigel; BD Biosciences, San Jose, CA, USA) was injected subcutaneously (500 \(\mu\)L/mouse) into the flank of 7-8 week-old female C57/B6 mice. The first group of mice were injected pure protein gelatin as control, others received protein gelatin mixed with either VEGF (250 ng), OPG (500 ng), or VEGF (250 ng) plus OPG (500 ng). After 7 or 8 days, plugs were resected and digested for 1 hour at \(37^\circ C\) with dispase (15 U/mL) in the presence of DNase (10,000 U/mL) to obtain single-cell suspensions. The total number of cells recovered from each plug was determined, and the cell suspensions were incubated with antibodies against CD45 and CD34 (clones 30-F11 and RAM34; eBioScience, San Diego, CA, USA), to distinguish between leukocytes and endothelial cells, respectively. Using flow cytometry the number of endothelial cells recruited into the plugs was determined.

**Statistical Analysis**

Data are mean \(\pm\) SD or SEM. The nonparametric Mann-Whitney \(U\) test was used to compare means from two independent groups. Pearson correlation coefficients were computed to investigate correlation between variables. A \(P\) value less than 0.05 indicated statistical significance. SPSS version 20.0 software (IBM, Armonk, NY, USA) for Windows (Microsoft, Redmond, WA, USA) was used for statistical analysis.

**RESULTS**

**ELISA Levels of OPG, VEGF, and MCP-1/CCL2 in Vitreous Samples From PDR Patients**

OPG was detected in 24 of 28 vitreous samples (86%) from nondiabetic control patients, as well as in 45 of 47 PDR samples (96%). Mean OPG level in PDR patients (364.5 \(\pm\) 163.3 pg/mL) was significantly higher than mean level in nondiabetic control patients (150.4 \(\pm\) 121.1 pg/mL) \((P < 0.0001)\). VEGF was detected in 17 of 28 samples (60.7%) and was significantly higher than mean level in nondiabetic control patients (38.3 \(\pm\) 81.1 pg/mL; \(P < 0.0001)\). MCP-1/CCL2 was detected in 25 of 28 samples (89%) from controls and in 45 of 47 samples (96%) from PDR patients. Mean MCP-1/CCL2 level in PDR patients (999.2 \(\pm\) 354 pg/mL) was...
FIGURE 5. (A) Osteoprotegerin mRNA is more abundant in retinas from subjects with diabetes. Expression of OPG mRNA was analyzed by using real-time quantitative RT-PCR in retinal RPE and neuroretina (NR). Osteoprotegerin mRNA expression was calculated after normalization of β-actin mRNA levels. Bars represent mean ± SD of the relative quantification (RQ) values obtained for mRNA levels in diabetic (D) (n = 10) and nondiabetic (C) (n = 10) donors. *P < 0.05 (Mann-Whitney U test). (B) Osteoprotegerin protein expression is upregulated in retinas from subjects with diabetes. Osteoprotegerin protein expression was determined by Western blot analysis on lysates of RPE and NR from representative diabetic (D) (n = 5) and nondiabetic (C) (n = 3) donors. Intensities of the protein bands around 50 kDa were determined in all samples (n = 10/group). Data (arbitrary units) are mean ± SD. *P < 0.05 (Mann-Whitney U test).
significantly higher than mean level in nondiabetic control patients (543 ± 271.9 pg/mL; P < 0.0001) (Fig. 1).

Significant positive correlations were found between vitreous fluid levels of OPG and levels of VEGF (r = 0.33; P = 0.006) and MCP-1/CCL2 (r = 0.41; P = 0.001). In contrast, the vitreous fluid levels of VEGF and MCP-1/CCL2 did not correlate.

**Western Blot Analysis of Vitreous Samples**

Using Western blot analysis, we confirmed that OPG was present in vitreous samples and that OPG expression was enhanced in PDR. Osteoprotegerin migrated at the position of its expected molecular weight and indeed, densitometric
analysis of the 50 kDa band demonstrated a significant increase in OPG expression in samples from PDR patients \((n = 16)\) compared to samples from nondiabetic control patients \((n = 8; P = 0.02)\) (Fig. 2).

**Immunohistochemical Analysis of Fibrovascular Epiretinal Membranes From Patients With PDR**

To identify the cellular source of vitreous fluid OPG, fibrovascular epiretinal membranes from patients with PDR were studied using immunohistochemical analysis. No staining was observed in the negative control slides (Fig. 3A). The levels of vascularization and proliferative activity in epiretinal membranes from patients with PDR were determined by immunodetection of the endothelial cell marker CD31. All membranes showed blood vessels that were positive for CD31 (Fig. 3B) with a mean of 33.7 ± 23.2 (range, 8 to 95) vessels per specimen. Leukocytes expressing the leukocyte common antigen CD45 (Fig. 3C) and spindle-shaped cells expressing the myofibroblast marker α-SMA (Fig. 3D) were detected in all membranes. Immunoreactivity for OPG was present in all membranes and was noted in vascular endothelial cells and stromal cells (Fig. 3E, 3F). Most of the OPG-positive stromal cells were spindle shaped (Fig. 3F). In serial sections, the distribution and morphology of spindle-shaped cells expressing OPG were similar to those of cells expressing α-SMA (Fig. 3D). Double immunohistochemistry showed that few stromal cells expressing OPG coexpressed CD45 (Fig. 3G). In comparison, there was no immunoreactivity for CD31 (Fig. 4A) and OPG (Fig. 4D) in epiretinal membranes from patients with PVR. On the other hand, all PVR membranes showed myofibroblasts expressing α-SMA (Fig. 4B) and leukocytes expressing CD45 (Fig. 4C).

The number of blood vessels that were immunoreactive for OPG ranged from 4 to 60, with a mean of 15.9 ± 17.3 per specimen. The number of OPG-positive stromal cells ranged from 3 to 105 cells, with a mean of 34.4 ± 36.9 per specimen.

Significant positive correlations were detected between the numbers of blood vessels expressing CD31 and the numbers of blood vessels \((r = 0.89; P < 0.0001)\) and stromal cells \((r = 0.56; P = 0.036)\) expressing OPG.

**Effect of Diabetes on Retinal OPG Expression in Humans and Rats**

OPG mRNA expression was higher in diabetic donors than in nondiabetic donors in both RPE and neuroretina \((P < 0.001\) for both comparisons) (Fig. 5A). Western blot analysis demonstrated that OPG protein expression was upregulated in diabetic retinas compared to nondiabetic control retinas in RPE as well as in neuroretina \((P < 0.001\) for both comparisons) (Fig. 5B). Immunostaining for OPG was observed in the cytoplasm of all...
retinal layers (Fig. 6A). In addition, double immunofluorescence analysis revealed that OPG was colocalized with the glial cell marker GFAP, the microglial cell marker Iba1 and the basal lamina marker collagen IV (Fig. 6B).

We quantified the expression of OPG in rat retinas by Western blot analysis. Densitometric analysis of the bands revealed that the expression of OPG did not differ significantly between nondiabetic controls and diabetic rat retinas after 4 and 12 weeks of acutely induced diabetes (Fig. 7).

**Human Retinal Microvascular Endothelial Cells Produce OPG In Vitro**

To confirm our findings that vascular endothelial cells in PDR fibrovascular epiretinal membranes express OPG, we investigated whether HRMEC could produce OPG. To reach that goal, we treated HRMEC for 72 hours with cytokines or growth factors known to be present in the ocular microenvironment of patients with PDR, in particular MCP-1/CCL2, VEGF, thrombin, IL-1β, and TNF-α. Unstimulated HRMEC did release a significant amount of OPG (about 45 pg/mL) in the culture medium (Fig. 8). The production of OPG by HRMEC was not upregulated in response to MCP-1/CCL2, VEGF or thrombin added as single stimulus or in combination. However, the cytokines IL-1β and TNF-α alone or in combination did increase the levels of OPG in the culture medium. In response to IL-1β plus TNF-α up to 15 ng/mL of OPG was detected by specific ELISA. Raising the glucose concentration of the culture medium to 25 mM did not affect spontaneous and stimulated release of OPG (data not shown).

**OPG Induces Human Retinal Microvascular Endothelial Cell Migration**

In order to investigate whether OPG affected HRMEC migration, real-time impedance-based recording of migration was performed using the xCELLigence system. HRMEC were stimulated with different doses of OPG, and migration was followed over 15 hours. Figure 9A shows the recorded cell indexes from one representative experiment. In total 4 experiments were performed and statistically evaluated. When results were expressed relative to the cell index of spontaneously migrating HRMEC, OPG significantly stimulated endothelial cell migration at 20 and 100 ng/mL (154 ± 17% and 153 ± 14%, respectively) (Fig. 9B). For comparison, VEGF, a potent inducer of endothelial cell migration, was also used as chemotactant. VEGF at 5 ng/mL significantly increased endothelial cell migration to 337 ± 42% (data not shown).

**OPG Activates Signal Transduction Pathways in Human Retinal Microvascular Endothelial Cells**

Next, we verified whether OPG was able to activate ERK1/2 and Akt signal transduction pathways. Similar to the angiogenic factor VEGF (50 ng/mL), OPG induced significant phosphorylation of these two kinases (Fig. 10). The minimal effective concentration of OPG, 300 ng/mL, was, however, at least 10-
fold higher than that of VEGF and OPG was not as efficacious as VEGF.

**OPG Induces In Vivo Angiogenesis**

Finally, we evaluated the angiogenic activity of OPG in protein gelatin plugs implanted subcutaneously in mice. In addition to control plugs (no extra stimulus; \( n = 12 \)), plugs containing 500 ng/mL OPG (\( n = 9 \)), 250 ng/mL VEGF (\( n = 10 \)), or OPG plus VEGF (\( n = 12 \)) were created. VEGF and the combination of VEGF plus OPG significantly increased the total number of cells recruited to the plugs (Fig. 11). Flow cytometric analysis demonstrated that an important part of the cells present in the plugs expressed CD34 in the absence of CD45, confirming that blood vessels (lined with endothelial cells) developed in the plugs, as could be observed macroscopically. In addition, OPG was able to enhance the effect of VEGF, suggesting that in eyes of diabetic patients where both OPG and VEGF are present, those endothelial growth factors cooperate in the induction of pathological neovascularization.

**DISCUSSION**

In the present study, we showed that OPG was significantly upregulated in the vitreous fluid from patients with PDR and in the retinas from subjects with diabetes mellitus. As yet it is unclear why the statistical differences observed in the levels of OPG in patients were not observed in the animal model. One possible explanation is the fact that the rat model represents short-term effects of an acute diabetogenic event, whereas in the patients, the disease evolved over much longer time intervals, eventually years. Using immunohistochemistry, we demonstrated that OPG protein was specifically produced by vascular endothelial cells, myofibroblasts and leukocytes in PDR fibrovascular epiretinal membranes. Our data are in line with those of others who have shown that OPG is expressed by endothelial cells, macrophages and fibroblasts in the microenvironment of tumors and inflammatory disorders.20–24

Although OPG was first described as a modulator of bone metabolism, an increasing number of reports now consider OPG also has a function in other biological systems, including promotion of angiogenesis and vasculogenesis.13,20 The angiogenic process requires activation and invasion of endothelial cells through their basement membrane, proliferation, migration and re-establishment of cell-cell contacts to form patent tubes. It has been demonstrated that OPG contributes to each of these events. Involvement of the heparin-binding domain of OPG in the proangiogenic activity of OPG was suggested.20,25–27 In addition, it was demonstrated that OPG enhanced the proangiogenic effect of VEGF.26 Similarly, in the present study, we demonstrate that OPG potentiated VEGF-induced angiogenesis in protein gelatin plugs implanted subcutaneously in mice. Additionally, OPG protects endothelial cells from apoptosis induced by growth factor withdrawal.28 OPG’s endothelial survival function appears to derive from its ability to inhibit TNF-related apoptosis inducing ligand (TRAIL)-mediated apoptosis.29 Furthermore, OPG stimulates endothelial colony-forming cell survival, migration, chemotaxis and
vascular cord formation suggesting that OPG is involved in vasculogenesis.\textsuperscript{30,31}

In the present study, we found a significant positive correlation between the vitreous fluid levels of OPG and those of the angiogenic and inflammatory biomarkers VEGF and MCP-1/CCL2. Using immunohistochemistry, we demonstrated a significant positive correlation between the level of vascularization in PDR epiretinal membranes and the number of blood vessels and stromal cells expressing OPG. Taken together, these findings suggest that upregulation of OPG in the ocular microenvironment of PDR patients might contribute to the progression of angiogenesis associated with PDR. To corroborate the findings at the cellular level, stimulation with OPG induced HRMEC migration, a key early step in angiogenesis. In addition, we showed that OPG can activate proliferative (ERK1/2) and survival (Akt) signaling pathways in HRMEC, further underscoring its role as a promoter of PDR angiogenesis. Our data are consistent with previous reports that demonstrated the ability of OPG to induce activation of the angiogenic signaling pathways ERK1/2 and Akt in endothelial cells.\textsuperscript{27,31,52}

Chronic low-grade subclinical inflammation plays a central role in the development of diabetic retinopathy.\textsuperscript{33} In this respect, it should be emphasized that OPG release by endothelial cells is significantly upregulated by the proinflammatory cytokines TNF-\(\alpha\), IL-1\(\beta\), and IL-1\(\beta\).\textsuperscript{18,26,34} as also shown in this study for HRMEC. These findings suggest that inflammation might be involved in diabetes-induced retinal endothelial structural damage by inducing OPG expression. Elevated levels of OPG have been observed in chronic inflammatory diseases,\textsuperscript{35–38} suggesting that OPG is a marker of inflammation. Previous reports demonstrated that OPG has proinflammatory properties by activation of classic nuclear factor-kappa B (NF-\(\kappa\)B) signaling.\textsuperscript{39} Furthermore, a pathologic role of OPG was also supported by an in vitro study showing that administration of OPG promoted the adhesion of leukocytes to endothelial cells. There is evidence that the heparin-binding domain of OPG is involved in mediating its pro-adhesive activity.\textsuperscript{40} OPG also stimulates upregulation of the endothelial adhesion molecules intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin in TNF-\(\alpha\)-activated endothelial cells and facilitates binding of monocytes.\textsuperscript{33} Because an abnormal increase of leukocyte/endothelial cell adhesion is considered an early step in diabetes-induced retinal vasculopathy,\textsuperscript{33} these data suggest that pathological increase of OPG in the ocular microenvironment of patients with PDR may contribute to the inflammatory status of the endothelium in PDR.

In conclusion, our findings suggest that OPG may be specifically involved in promoting the progression of pathologic neovascularization in the ocular microenvironment of PDR patients. Therapeutic strategies aimed to decrease OPG levels may be suitable for improving the vascular function in diabetic retina.

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