Regulation of Angiogenesis in Diabetic Retinopathy

Possible Balance Between Vascular Endothelial Growth Factor and Endostatin

Hidetaka Noma, MD; Hideharu Funatsu, MD; Hidetoshi Yamashita, MD; Shigehiko Kitano, MD; Hiromu K. Mishima, MD; Sadao Hori, MD

Objective: To investigate the mechanisms of regulation between vascular endothelial growth factor (VEGF) as a stimulator and endostatin as an inhibitor of angiogenesis in diabetic retinopathy (DR).

Methods: One hundred fifty-nine eyes of 120 diabetic patients were studied. Concentrations of VEGF and endostatin in vitreous fluid and aqueous humor, obtained from the eyes during ocular surgery, were measured by enzyme-linked immunosorbent assay. The severity of DR was quantified according to the Early Treatment Diabetic Retinopathy Study retinopathy severity scale; fundus findings, including soft exudates, intraretinal microvascular abnormalities, venous abnormalities, new vessels elsewhere, new vessels on the disc, vitreous hemorrhage, and retinal detachment, were graded and evaluated. Concentrations of VEGF and endostatin in plasma were also measured by enzyme-linked immunosorbent assay.

Main Outcome Measures: Concentrations of VEGF and endostatin in vitreous fluid and plasma. The correlations among the clinical records and the levels of VEGF and endostatin were analyzed statistically.

Results: The concentrations of VEGF in aqueous humor and vitreous fluid were significantly correlated with the severity of DR ($\rho=0.447$, $P<.001$ and $\rho=0.363$, $P=.007$, respectively). The concentrations of endostatin in aqueous humor and vitreous fluid were also significantly correlated with the severity of DR ($\rho=0.302$, $P<.001$ and $\rho=0.344$, $P=.009$, respectively). The slope of the regression line between the VEGF and endostatin concentrations in vitreous fluid differed significantly between active DR and quiescent DR ($P=.04$). The concentrations of VEGF and endostatin in the eyes were not correlated with those in the plasma.

Conclusions: These results show that both VEGF and endostatin are correlated with angiogenesis in DR. Our study suggests that the regulation mechanism between VEGF and endostatin is associated with the activity of DR and may be a good candidate to develop useful therapeutic agents for proliferative DR.

Arch Ophthalmol. 2002;120:1075-1080

NEW VESSEL formation in diabetic retinopathy (DR) causes visual loss with vitreous hemorrhage, retinal detachment, and neovascular glaucoma.1,2 Angiogenesis is a complex multistep process. Various cytokines and growth factors are considered to be involved in these processes and the pathogenesis of angiogenesis.3,5 However, not all the mechanisms involved in the process of neovascularization in proliferative DR can be explained in terms of only angiogenic stimulators. It is hypothesized that the net balance between angiogenic stimulators and inhibitors regulates the switching of the angiogenic process.5-8 That is, a net balance of inhibitors over activators would maintain the switch in the off position, whereas a shift to an excess of activating stimuli would turn on angiogenesis. Vascular endothelial growth factor (VEGF) acts as an endothelial cell mitogen9,10 in vitro and induces increased vascular permeability11 and angiogenesis in vivo. Intraocular VEGF concentrations are increased during the periods of active intraocular neovascularization in patients with proliferative DR.12

Many endogenous inhibitors of angiogenesis, including endostatin,13 thrombospondin,14,15 interferon α and β, prolactin, platelet factor 4,16-18 and angioptatin,19 have been reported. Endostatin is an angiogenic inhibitor produced by hemangioendothelioma.13 Endostatin specifically inhibits endothelial cell proliferation and potently inhibits angiogenesis and tumor growth.13 Endostatin inhibits the VEGF-stimulated migration of umbilical vein endothelial cells.20 Based on

From the Departments of Ophthalmology, Diabetes Center, Tokyo Women's Medical University, Tokyo (Drs Noma, Funatsu, and Kitano), Hiroshima University Medical School, Hiroshima (Drs Noma and Mishima), and Yamagata University Medical School, Yamagata (Dr Yamashita), and Tokyo Women's Medical University (Dr Hori), Japan.

©2002 American Medical Association. All rights reserved.
**PARTICIPANTS AND METHODS**

**STUDY PARTICIPANTS**

Samples of aqueous humor and vitreous fluid were obtained from 139 eyes of 120 diabetic patients whose mean (SD) age was 62.0 (12.3) years (range, 24-88 years). The patients included 77 men and 43 women. The mean (SD) duration of diabetes mellitus was 15.7 (9.5) years (range, 9-30 years). Patients were excluded if they had undergone previous intraocular surgery or had a history of branch retinal vein occlusion and uveitis. All procedures conformed to the Declaration of Helsinki for research involving human subjects. Ethics committee approval was obtained, and all participants gave informed consent.

The activity of DR is classified into active and quiescent. If there are extensive changes, including active neovascularization and proliferative membrane, fresh vitreous hemorrhage, and progressive retinal detachment, it is classified as active. If it becomes silent on photococoagulation, even with remaining neovascularization and proliferative membrane, it is classified as quiescent.

**SAMPLE COLLECTION**

At the start of eye surgery, a sample of undiluted aqueous humor (100-200 µL) was manually aspirated into a disposable tuberculin syringe, and a sample of undiluted vitreous fluid (300-500 µL) was aspirated using a vitreous cutter (under air flow), transferred immediately to a sterile tube, frozen immediately at −5°C, and then stored in a deep freezer at −80°C. Blood samples were collected simultaneously and centrifuged at 3000 g for 5 min to obtain serum and then aliquoted and stored at −80°C until they were assayed.

**MEASUREMENT OF VEGF AND ENDOSTATIN**

Concentrations of VEGF and endostatin were measured by an enzyme-linked immunosorbent assay using immunoassay kits for human VEGF (R&D Systems, Minneapolis, Minn) and human endostatin (Cytimmune Sciences, Baltimore, Md), respectively, according to the manufacturer’s standard protocol. The results ensured that the levels of factors in intraocular fluid and plasma samples were within the detectable range using these assays. The minimum detectable concentrations (sensitivity) using the assay kits were 15.6 pg/mL for VEGF and 0.95 ng/mL for endostatin.

**FINDINGS AND GRADING PROCEDURES OF DR**

The preoperative and operative findings were recorded. Clinical data, including the severity of DR, were obtained by the surgeon using standardized forms at the time of surgery and were confirmed by standardized fundus color photography and fluorescein angiography performed within 3 days after the operation. The severity of DR was graded according to the modified Early Treatment Diabetic Retinopathy Study (ETDRS) retinopathy severity scale. In particular, the severity of soft exudates, intraretinal microvascular abnormalities (IRMA), venous beading, venous loops, new vessels elsewhere (NVE), new vessels on the disc (NVD), fibrous proliferation elsewhere (FPE), vitreous hemorrhage, and retinal detachment were graded according to the ETDRS system.

**STATISTICAL ANALYSIS**

Statistical analysis was performed with SAS/STAT statistical software (SAS Institute Inc, Cary, NC; changes and enhancements through release 6.12). Results are presented as mean±SD or geometric mean±SD for data shown on the logarithmic scale. To determine the relationship between angiogenic factors and the ETDRS retinopathy severity, Spearman rank-order correlation coefficient was applied. To test the heterogeneity of slopes of 2 linear regression lines, analysis of covariance with interaction was used. In this model, variables were analyzed on the logarithmic scale because of skewed distribution. Two-tailed P values of less than .05 were considered to indicate statistical significance.

**RESULTS**

**ANGIOGENIC FACTORS AND SEVERITY OF DR**

The concentrations of VEGF in the aqueous humor (geometric mean, 223.3 pg/mL) and vitreous fluid (geometric mean, 1291 pg/mL) were significantly correlated with the severity of DR (ρ=0.447, P<.001 and ρ=0.363, P=.007, respectively) (Figure 1A and B). The concentrations of endostatin in the aqueous humor (mean, 2.6 ng/mL) and vitreous fluid (mean, 4.0 ng/mL) were also significantly correlated with the severity of DR (ρ=0.302, P<.001 and ρ=0.344, P=.009, respectively) (Figure 2A and B).

**ANGIOGENIC FACTORS AND SEVERITY OF FUNDUS FINDINGS**

The concentration of VEGF in vitreous fluid was significantly correlated with the grades of soft exudate, IRMA, venous beading, venous loops, NVE, NVD, FPE, and vitreous hemorrhage (Table). The concentration of endostatin in vitreous fluid was significantly correlated with the grades of NVE, FPE, and retinal detachment (Table).

**VEGF CORRELATIONS**

VEGF and Endostatin in Eyes

With Active or Quiescent DR

The VEGF levels were higher in the vitreous fluid of active DR than in the vitreous fluid of quiescent DR. The
endostatin levels were lower in the vitreous fluid of active DR than in the vitreous fluid of quiescent DR. On the other hand, in the vitreous fluid of quiescent DR, the concentration of VEGF was low but the concentration of endostatin was high.

The recurrence equations were as follows: endostatin = $1.538754 + 0.003985 \times$ VEGF for quiescent DR and endostatin = $1.619433 + 0.001151 \times$ VEGF for active DR. The slope of the regression line between the VEGF and endostatin concentrations in vitreous fluid differed significantly between active DR and quiescent DR ($P=.04$) (Figure 3).

Concentrations of VEGF and Endostatin

There were significant correlations between the concentration of VEGF and endostatin in the aqueous humor.
and vitreous fluid \( (\rho=0.320, \ P=0.004 \text{ and } \rho=0.374, \ P=0.006, \text{ respectively}) \) (Figure 4A and B).

**Plasma Concentrations of VEGF and the Severity of DR**

There was no significant correlation between the plasma concentration of VEGF or endostatin and the severity of DR (data not shown).

**Concentrations of VEGF and Endostatin in the Ocular Fluid and Plasma**

The concentration of VEGF in neither the aqueous humor (223.3 pg/mL) nor the vitreous fluid (1291 pg/mL) was significantly correlated with that in plasma (66.3 pg/mL). As for endostatin, no significant relationship was found between its concentration in aqueous humor (2.6 ng/mL) and plasma (7.4 ng/mL), but a significant relationship was found between its concentration in vitreous fluid (4.0 ng/mL) and plasma (\( \rho=0.054, \ P=0.72 \text{ and } \rho=0.377, \ P<0.05 \), respectively).

**COMMENT**

We obtained the following findings in this study. First, in patients with DR, endostatin was detected in aqueous humor and vitreous fluid. Second, the endostatin and VEGF concentrations in aqueous humor and vitreous fluid were correlated with the severity of DR. Third, some of the patients with severe DR showed a high endostatin concentration in vitreous fluid, but others showed a low concentration. Fourth, the slope of the regression line between the VEGF and endostatin concentrations in vitreous fluid differed significantly between active DR and quiescent DR.

There was a significant positive correlation between the severity of DR and the VEGF concentration in the aqueous humor and vitreous fluid in the present study. These results were consistent with the findings of previous reports. The VEGF concentration in the vitreous fluid was positively correlated with the grades of soft exudates, IRMA, venous beading, venous loops, NVE, NVD, FPE, and vitreous hemorrhage (Table). These results suggest that VEGF stimulates angiogenesis in the pathology of DR. Our results and the previous reports suggest that VEGF is associated with enhanced vascular permeability, vascular occlusion, and angiogenesis.

The severity of DR was also positively correlated with the endostatin concentrations in both aqueous humor and vitreous fluid. Of the fundus findings, the grades of NVE, FPE, and retinal detachment were positively correlated with the endostatin concentration in vitreous fluid (Table). In the aqueous humor and vitreous fluid, the VEGF concentration was positively correlated with the endostatin concentration. These results suggest that endostatin expression is correlated with VEGF expression.

The theory that the balance between stimulators and inhibitors is critical in the process of tumor angiogenesis has been proposed. We consider endostatin to be an angiogenic inhibitor associated with the pathogenesis of proliferative DR (PDR). Endostatin is an angiogenic inhibitor produced by hemanigioendothelioma. Endostatin specifically inhibits endothelial cell proliferation, potently inhibiting angiogenesis and tumor growth. Endostatin has also been reported to inhibit VEGF-stimulated endothelial cell proliferation and migration. Thus, there is a possibility that endostatin inhibits angiogenesis in PDR, but this possibility has not yet been evaluated. In this study, the endostatin concentration in intraocular fluid and blood samples was evaluated.

Although the endostatin concentration in both aqueous humor and vitreous fluid showed a significant positive correlation with the severity of DR, the endostatin concentration in aqueous humor was high even in some patients with mild DR and varied widely in patients with...
severe DR, showing a uniform distribution independent of the severity of DR. The endostatin concentration in vitreous fluid varied more widely as the severity of DR increased. This indicates that the endostatin concentration in vitreous fluid is high in some patients with severe DR but low in others with severe DR. To clarify differences between patients with a high endostatin concentration and those with a low concentration, the net balance between VEGF and endostatin was evaluated in association with the activity of DR. The activity of DR was classified as active (highly active neovascularization and proliferative membrane, fresh vitreous hemorrhage, and progression to retinal detachment) or quiescent (silent on photocoagulation even with remaining neovascularization and proliferative membrane). In addition, DR was active in patients with a high VEGF concentration and a low endostatin concentration in vitreous fluid. However, DR was quiescent in patients with a low VEGF concentration and a high endostatin concentration in vitreous fluid. Retinopathy was frequently active in patients with high VEGF and high endostatin concentrations but frequently quiescent in those with low VEGF and low endostatin concentrations. The slope of the regression line between the VEGF and endostatin concentrations in vitreous fluid differed significantly between active DR and quiescent DR (P<.05, Figure 3). These findings suggest that the activity of DR rather than the severity of DR more accurately reflects the effects of angiogenic stimulators and inhibitors on DR. In addition, 4 patients with a high endostatin concentration had quiescent DR despite a high VEGF concentration, and 1 patient with a low endostatin concentration had active DR despite a low VEGF concentration. Although these results suggest the promotion of neovascularization by VEGF and its inhibition by endostatin, their direct mechanisms could not be clarified in this study. However, in DR and tumors, the net balance of factors seems to affect the eyes.

It was reported that other angiogenesis stimulators and inhibitors possibly play a role in angiogenesis in PDR. The lower vitreous levels of pigment epithelium–derived factor or angiotatin may be related to the angiogenesis in PDR and result in active PDR. It is possible that not only endostatin but also other angiogenesis inhibitors play a role in antiangiogenesis in PDR. Further investigation is needed to resolve the mechanisms of angiogenesis in PDR.

There was no significant correlation between the plasma VEGF or endostatin concentration and the severity of DR or between the VEGF concentration in the aqueous humor or vitreous fluid and the plasma VEGF concentration. The VEGF concentration in vitreous fluid (1291 pg/mL) was much higher than in plasma (66.3 pg/mL). These results suggest that VEGF is produced in the eyes. VEGF has been reported to be involved in the destruction of the blood-retinal barrier. These results suggest that leakage of endostatin from the blood to the vitreous body occurs because of VEGF-induced destruction of the blood-retinal barrier. Since no difference was observed in the endostatin concentration according to the presence or absence of retinal photocoagulation, endostatin may not be produced exclusively in the eyes (data not shown). However, the range of the plasma endostatin concentration was narrow, but that of the vitreous endostatin concentration was wide. In addition, the intraocular endostatin concentration was higher than

**Figure 4.** Correlation between the concentrations of vascular endothelial growth factor (VEGF) and endostatin. A, There was a significant correlation between the concentrations of VEGF and endostatin in aqueous humor samples from patients with diabetes (n=77, p=0.320, P=.004). B, There was a significant correlation between the concentrations of VEGF and endostatin in vitreous fluid samples from patients with diabetes (n=52, p=0.374, P=.006).
the plasma endostatin concentration in 10 patients, suggesting that endostatin is also produced in the eyes. Whether endostatin is produced in the eyes could not be determined in this study. Further studies will be necessary to evaluate the tissue origin of endostatin.

In conclusion, we found that the levels of endostatin and VEGF concentrations are correlated with the severity of DR, and the slope of the regression line between the VEGF and endostatin concentrations in vitreous fluid differs significantly between active DR and quiescent DR.

Submitted for publication July 10, 2001; final revision received March 28, 2002; accepted April 24, 2002.

This study was supported by Health Science Research grants (10060101) from the Ministry of Health and Welfare, Research on Eye and Ear Sciences, Immunology, Allergy and Organ Transplantation in Japan (Tokyo).

We thank Erika Shimizu, MD, Yasuyuki Konno, MD, Kozue Ohara, MD, Kaori Sekimoto, MD, Rie Takeda, MD, Koji Makita, MD, Kensuke Haruyama, MD, Shinko Nakamura, MD, and Maho Hirai, MD, for assistance in the collection of vitreous samples and ophthalmological examinations. We thank Katsunori Shimada (Department of Bio-statistics, STATZ Corporation, Tokyo) for assistance with statistical analysis.

Corresponding author and reprints: Hideharu Funatsu, MD, Department of Ophthalmology, Diabetes Center, Tokyo Women’s Medical University, 8-1, Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan (e-mail: tfunatsu@nifty.com).

REFERENCES


©2002 American Medical Association. All rights reserved.