

ORIGINAL ARTICLE

Erythropoietin as a Retinal Angiogenic Factor in Proliferative Diabetic Retinopathy

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ABSTRACT

BACKGROUND

Although vascular endothelial growth factor (VEGF) is a primary mediator of retinal angiogenesis, VEGF inhibition alone is insufficient to prevent retinal neovascularization. Hence, it is postulated that there are other potent ischemia-induced angiogenic factors. Erythropoietin possesses angiogenic activity, but its potential role in ocular angiogenesis is not established.

METHODS

We measured both erythropoietin and VEGF levels in the vitreous fluid of 144 patients with the use of radioimmunoassay and enzyme-linked immunosorbent assay. Vitreous proliferative potential was measured according to the growth of retinal endothelial cells in vitro and with soluble erythropoietin receptor. In addition, a murine model of ischemia-induced retinal neovascularization was used to evaluate erythropoietin expression and regulation in vivo.

RESULTS

The median vitreous erythropoietin level in 73 patients with proliferative diabetic retinopathy was significantly higher than that in 71 patients without diabetes (464.0 vs. 36.5 mIU per milliliter, $P < 0.001$). The median VEGF level in patients with retinopathy was also significantly higher than that in patients without diabetes (345.0 vs. 3.9 pg per milliliter, $P < 0.001$). Multivariate logistic-regression analyses indicated that erythropoietin and VEGF were independently associated with proliferative diabetic retinopathy and that erythropoietin was more strongly associated with the presence of proliferative diabetic retinopathy than was VEGF. Erythropoietin and VEGF gene-expression levels are up-regulated in the murine ischemic retina, and the blockade of erythropoietin inhibits retinal neovascularization in vivo and endothelial-cell proliferation in the vitreous of patients with diabetic retinopathy in vitro.

CONCLUSIONS

Our data suggest that erythropoietin is a potent ischemia-induced angiogenic factor that acts independently of VEGF during retinal angiogenesis in proliferative diabetic retinopathy.

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N Engl J Med 2005;353:782-92.
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PATHOLOGIC GROWTH OF NEW BLOOD vessels is a common final pathway in ocular neovascular diseases, such as proliferative diabetic retinopathy, that often result in catastrophic loss of vision. Vascular endothelial growth factor (VEGF) is a primary angiogenic factor that mediates such ischemia-induced retinal neovascularization. VEGF levels are elevated in the vitreous fluid of patients with proliferative diabetic retinopathy, and VEGF induces proliferation in vascular endothelial cells *in vitro*.¹ Although inhibition of VEGF reduces retinal neovascularization,^{2,3} it does not completely inhibit ischemia-driven retinal neovascularization. Thus, the involvement of other angiogenic factors in this process seems likely.

The glycoprotein erythropoietin stimulates the formation of red cells by enhancing both their proliferation and their differentiation and by preventing apoptotic death of erythropoietin-responsive erythroid precursor cells.⁴⁻⁶ A major signal that regulates the production of erythropoietin in these tissues is hypoxia, and the brain has a paracrine system involving erythropoietin and erythropoietin receptors, suggesting that erythropoietin contributes to the survival of neurons by protecting them from ischemic damage.⁷⁻⁹ Furthermore, erythropoietin shows angiogenic activity in vascular endothelial cells, stimulating proliferation, migration, and angiogenesis *in vitro*, probably by means of the erythropoietin receptor expressed in those cells.^{10,11} Such angiogenic activity involves several signal-transduction cascades such as extracellular signal-regulated kinase, Janus kinase 2 (known as JAK2), and signal transducer and activator of transcription 5 (STAT5).¹²⁻¹⁵ Moreover, the inhibition of erythropoietin by soluble erythropoietin receptor abrogates angiogenesis *in vivo*.^{16,17}

Since erythropoietin is an ischemia-induced paracrine factor that promotes angiogenesis, we wished to identify its potential role during retinal angiogenesis in proliferative diabetic retinopathy. Therefore, we examined *in vitro* the expression and function of erythropoietin in the vitreous fluid of patients with proliferative diabetic retinopathy and evaluated the role of erythropoietin in an *in vivo* experimental model of retinal angiogenesis. Our data provide strong evidence that erythropoietin is a potent retinal angiogenic factor independent of VEGF and is capable of stimulating ischemia-induced retinal angiogenesis in proliferative diabetic retinopathy.

METHODS

PREPARATION OF VITREOUS-FLUID SAMPLES AND ANALYSIS OF ERYTHROPOIETIN AND VEGF LEVELS

We conducted this study in accordance with the Declaration of Helsinki and received institutional approval from the review committees of Kyoto University Hospital and Otsu Red Cross Hospital. Patients with proliferative diabetic retinopathy or nondiabetic ocular diseases who were seen consecutively for treatment with pars plana vitrectomy at these centers were approached for participation from June 1997 through September 2004. All patients provided written informed consent. Samples of undiluted vitreous fluid were harvested from the eyes of participating patients who had proliferative diabetic retinopathy or nondiabetic ocular diseases. None of the patients with nondiabetic ocular diseases had diabetes mellitus. Proliferative diabetic retinopathy was classified clinically as active neovascularization if there were perfused, multi-branching iridic or preretinal capillaries and as quiescent if previously documented active prolifer-

Table 1. Characteristics of the Patients.*

Characteristic	Proliferative Diabetic Retinopathy (N=73)	Nondiabetic Ocular Diseases (N=71)
Age — yr	60.8±10.1	61.4±14.1
Female sex — no. (%)	34 (47)	47 (66)
Duration of diabetes — yr	12.3±9.0	—
Glycosylated hemoglobin — %	7.6±1.6	—
Subgroups — no. (%)		
Active	52 (71)	—
Quiescent	21 (29)	—
Idiopathic epiretinal membrane	—	8 (11)
Inflammation†	—	14 (20)
Idiopathic macular hole	—	22 (31)
Rhegmatogenous retinal detachment	—	22 (31)
Other‡	—	5 (7)

* Plus-minus values are means ±SD. Dashes denote not applicable.

† The inflammation subgroup includes 3 patients with proliferative vitreoretinopathy and 11 with uveitis.

‡ Other includes two patients with retinal macroaneurysms, two with Terson's syndrome, and one with choroidal neovascular membrane.

ation had regressed fully or if there were only non-perfused, gliotic vessels or fibrosis.¹

Blood samples were collected if available. Erythropoietin levels were measured with the use of radioimmunoassay (Recombinagen Epo kit, Diagnostic Products Corporation). VEGF levels were measured with the use of an enzyme-linked immunosorbent assay (ELISA) (R&D Systems).

CELL CULTURE, WESTERN BLOT ANALYSIS, AND ASSAYS OF CELL GROWTH

Total protein levels were obtained from primary cultures of bovine retinal microvascular endothelial cells (BRECs),¹⁸ and the expression of specific antigens was assessed by Western blot analysis¹⁹ with primary antibodies against phospho-p44/p42 (New England Biolabs), extracellular signal-regulated kinase 1 (Santa Cruz Biotechnology), phospho-STAT5 (Upstate Biotechnology), and STAT5 (Santa Cruz Biotechnology).

BRECs were plated separately (700 cells per well) in 96-well culture plates coated with collagen type I.¹ The next day, human recombinant erythropoietin (0.1 to 50 IU per milliliter) (Kirin), human recombinant VEGF (10 ng per milliliter) (Genzyme), or vitreous fluid (final volume, 20 percent) was added in conjunction with phosphate-buffered saline, soluble erythropoietin receptor (25 μ g per milliliter), soluble Flt-1-Fc chimera (2.5 μ g per milliliter) (Genzyme), both proteins, or heat-denatured soluble erythropoietin receptor (25 μ g per milliliter). Soluble erythropoietin receptor was prepared as previously described.²⁰ After four days, cell growth was assayed with the use of 5-bromo-2'-deoxyuridine during DNA synthesis. The absorbance of each sample was measured with an ELISA reader and compared with the absorbance of control samples stimulated with phosphate-buffered saline. We performed the assay of cell growth in triplicate for the samples from each patient.

REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was prepared from the retinas of mice with the use of an acid guanidinium thiocyanate-phenol-chloroform method. This was followed by synthesis of complementary DNA and a real-time polymerase chain reaction (PCR).²¹

ISCHEMIA-INDUCED RETINAL NEOVASCULARIZATION IN A MURINE MODEL

All animals were handled according to the Association for Research in Vision and Ophthalmology

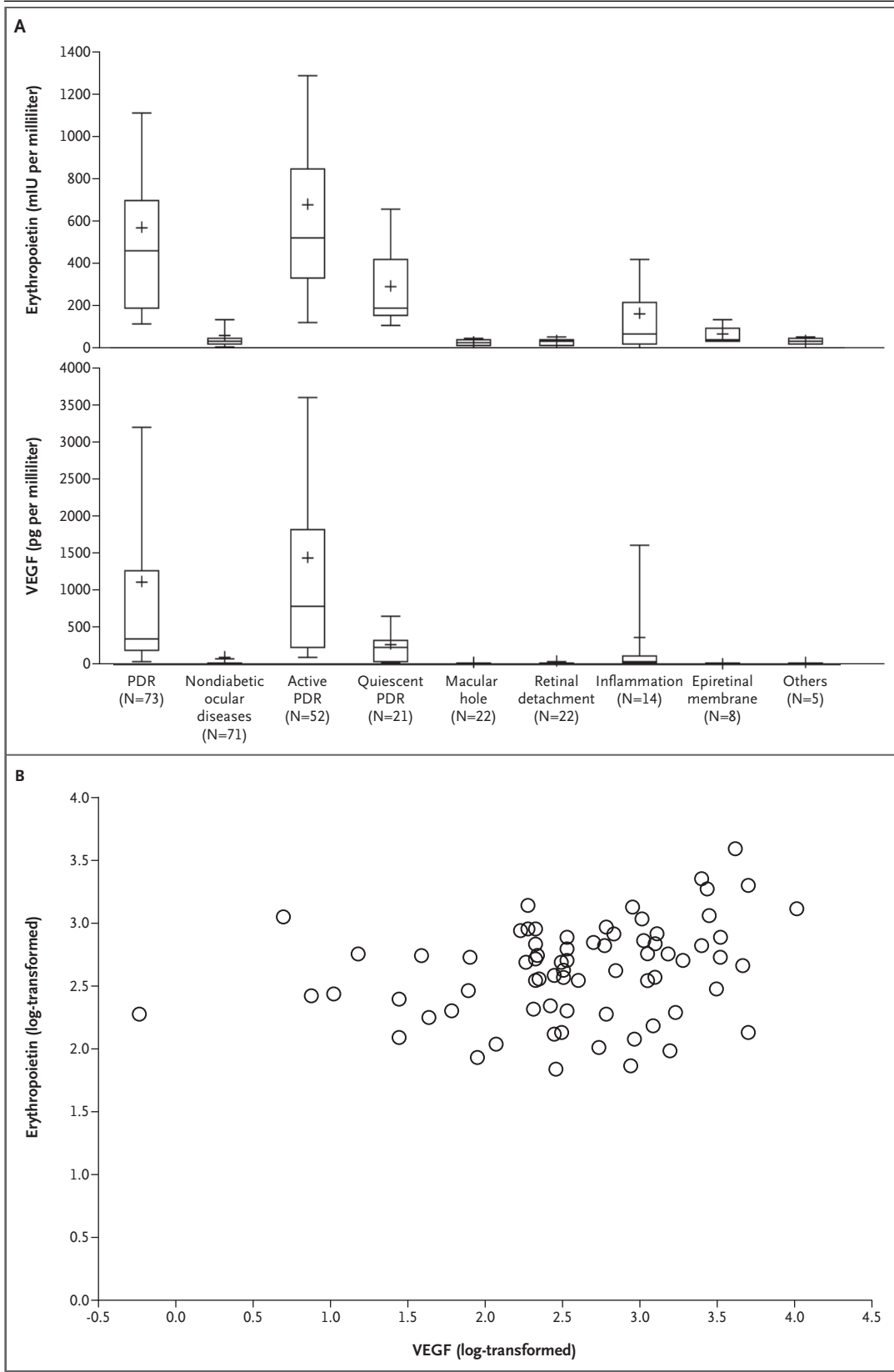
Figure 1 (facing page). Vitreous Levels of Erythropoietin and Vascular Endothelial Growth Factor (VEGF).

In Panel A, box-and-whisker plots show the levels of erythropoietin and VEGF for patients with proliferative diabetic retinopathy (PDR) and for patients with nondiabetic ocular diseases. The plots also show the levels in the subgroups with active and quiescent proliferative diabetic retinopathy and those in the following subgroups of nondiabetic ocular diseases: idiopathic macular hole, rhegmatogenous retinal detachment, inflammation, idiopathic epiretinal membrane, or other diseases. The lower and upper whiskers correspond to the 10th and 90th percentiles, respectively. The line in the box corresponds to the median value, and the plus sign (+) represents the mean value. Panel B shows a scatter plot for the correlation between log-transformed vitreous VEGF levels and vitreous erythropoietin levels in patients with proliferative diabetic retinopathy.

Statement for the Use of Animals in Ophthalmic and Vision Research (www.arvo.org/AboutARVO/animalst.asp). Litters of seven-day-old C57BL/6J mice were exposed to oxygen (mean [\pm SD], 75 \pm 2 percent) for five days and then returned to room air on day 12.²² Mice of the same age, kept in room air, served as controls. A 0.5- μ l solution containing various concentrations of either soluble erythropoietin receptor, soluble Flt-1-Fc, both proteins, or heat-denatured soluble erythropoietin receptor was injected into the vitreous of one eye on day 12 and day 14.² As a control, an equivalent volume and concentration of human IgG was injected into the contralateral eye on the same days. On day 19, neovascular quantification was performed in a double-masked manner.²

STATISTICAL ANALYSIS

The primary objective of this study was to determine whether erythropoietin is an angiogenic factor independent of VEGF in patients with proliferative diabetic retinopathy. For primary analyses, we performed a two-sample t-test to compare the level of erythropoietin in the group of patients who had proliferative diabetic retinopathy with the level in the group with nondiabetic ocular diseases. We calculated the Pearson correlation coefficient between the erythropoietin and VEGF levels in the proliferative diabetic retinopathy group; data on erythropoietin and VEGF levels were transformed with the use of common base-10 logarithms. We also performed the Mann-Whitney rank-sum test and calculated the Spearman rank-correlation coefficient to confirm the results of parametric analyses. To demonstrate and quantify the independent associ-



ations of erythropoietin and VEGF with proliferative diabetic retinopathy, we performed multivariate logistic-regression analyses with disease status (proliferative diabetic retinopathy or nondiabetic ocular diseases) as the response variable and with four covariates — erythropoietin, VEGF, age, and sex. In vitro data were analyzed with the use of one-way analysis of variance followed by Fisher's least-significant-difference test. For evaluation of in vivo retinal angiogenesis, the paired t-test or the Mann-Whitney rank-sum test was used for quantitative data. All reported P values are two-sided.

To detect an approximately 100 percent mean difference in erythropoietin between the group of patients with proliferative diabetic retinopathy and the group with nondiabetic ocular diseases with the use of a two-sample t-test on log-transformed data, with a two-sided alpha of 0.05 and a power of 95 percent, a sample size of 72 was required for each group. The variance of the log-transformed erythropoietin value was set to be 0.24, which was suggested as a result of preliminary data from five patients with proliferative diabetic retinopathy. To analyze the correlation between erythropoietin and VEGF for 72 patients with proliferative diabetic retinopathy, we had a power of 0.94 when we used a test of no correlation on the basis of the Fisher transformation of the Pearson correlation coefficient, resulting in a true correlation value of 0.4.

RESULTS

VITREOUS ERYTHROPOIETIN AND VEGF LEVELS

Samples of undiluted vitreous fluid were harvested from the eyes of 73 patients with proliferative diabetic retinopathy and 71 patients with nondiabetic ocular diseases, including idiopathic epiretinal membrane, proliferative vitreoretinopathy, uveitis, idiopathic macular hole, rhegmatogenous retinal detachment, retinal macroaneurysms, Terson's syndrome, and choroidal neovascular membrane (Table 1). Samples of aqueous humor harvested from seven patients with uveitis were included.

The vitreous erythropoietin and VEGF levels were significantly higher in the patients with proliferative diabetic retinopathy than in those with nondiabetic ocular diseases (Fig. 1A). The median erythropoietin level was 464.0 mIU per milliliter (range, 64.5 to 3720.0) in the patients with proliferative diabetic retinopathy and 36.5 mIU per milliliter (range, 0.4 to 698.0) in the patients with nondiabetic ocular diseases ($P<0.001$). The median

VEGF level was 345.0 pg per milliliter (range, 0 to 10,600.0) in the group with proliferative diabetic retinopathy and 3.9 pg per milliliter (range, 0 to 2130.0) in the nondiabetic group ($P<0.001$). Clearly, the proportion of patients with proliferative diabetic retinopathy, which corresponds to the response variable in the multivariate logistic-regression analyses, was higher in higher quartiles of erythropoietin and VEGF levels (Table 2).

For exploratory purposes, we also examined the erythropoietin and VEGF levels in subgroups of patients with various stages of proliferative diabetic retinopathy as compared with subgroups of patients with various nondiabetic ocular diseases (Fig. 1A). The level of erythropoietin was significantly higher in the patients with active as compared with quiescent proliferative diabetic retinopathy ($P=0.001$). The level of erythropoietin was significantly higher in the patients with proliferative diabetic retinopathy than in the patients in the nondiabetic subgroups ($P<0.001$). Among the patients with nondiabetic ocular diseases, those with inflammation had slightly higher levels of erythropoietin than did those with the other diseases.

A scatter plot of log-transformed levels of erythropoietin and VEGF in the patients with proliferative diabetic retinopathy indicated a weak correlation (Fig. 1B). The Pearson correlation coefficient was 0.29 ($P=0.01$). The corresponding value for patients with active proliferative diabetic retinopathy was 0.14 ($P=0.32$), and that for patients with quiescent proliferative diabetic retinopathy was 0.28 ($P=0.23$). Similar results were obtained from rank-based nonparametric analyses (data not shown).

Blood samples were collected from 36 patients with proliferative diabetic retinopathy and 42 with nondiabetic ocular diseases. The median plasma level of erythropoietin was 18.7 mIU per milliliter (range, 7.0 to 43.4) in the group with proliferative diabetic retinopathy and was slightly lower than that in the nondiabetic group (22.4 mIU per milliliter; range, 5.6 to 63.6). The Pearson correlation coefficient for the plasma and the vitreous erythropoietin levels in patients with proliferative diabetic retinopathy was -0.16 ($P=0.14$).

In multivariate logistic-regression analyses, the association of erythropoietin and VEGF with proliferative diabetic retinopathy was initially assessed with the use of untransformed values but was found to fit substantially better with the use of log transformations to make the erythropoietin and VEGF distributions symmetric. Neither age ($P=0.39$) nor

sex ($P=0.74$) was significantly associated with proliferative diabetic retinopathy (Table 3), and the two covariates were thus dropped from the model. When we fitted only log-transformed erythropoietin and VEGF, we observed that both were significantly associated with proliferative diabetic retinopathy, but erythropoietin more strongly (Table 3).

TREATMENT WITH SOLUBLE ERYTHROPOIETIN RECEPTOR TO ATTENUATE ERYTHROPOIETIN INTRACELLULAR SIGNALING

Erythropoietin (10 IU per milliliter) increased phosphorylation of intracellular signaling substrates of STAT5 and 42/44 mitogen-activated protein kinase 15 minutes after stimulation of BRECs. This increase was inhibited by the addition of soluble erythropoietin receptor at a concentration 2500 times as great as that of erythropoietin (Fig. 2A).

EFFECT OF ERYTHROPOIETIN, VEGF, AND VITREOUS FLUID ON THE GROWTH OF RETINAL ENDOTHELIAL CELLS

The growth of BRECs was stimulated in a dose-dependent manner after exposure to erythropoietin, with a maximal cell growth observed at 20 IU of erythropoietin per milliliter (absorbance, 1.63 ± 0.19 times as high as that of control samples stimulated with phosphate-buffered saline; $P < 0.001$) (Fig. 2B). VEGF (10 ng per milliliter) also stimulated cell growth (absorbance, 1.90 ± 0.29 ; $P < 0.001$). Vitreous samples from eight patients with proliferative diabetic retinopathy stimulated the growth of BRECs, which was significantly inhibited by soluble erythropoietin receptor (25 μ g per milliliter) in all cases (range of inhibition, 22 to 74 percent). Soluble Flt-1-Fc also inhibited cellular growth in each case (range of inhibition, 22 to 85 percent) (Fig. 2C), and the mean inhibitory effect of erythropoietin and VEGF blockade on cellular growth, expressed as a percentage of the level induced by the vitreous fluid from each of the eight patients, was 56, 54, and 16 percent after the addition of soluble erythropoietin receptor, soluble Flt-1-Fc, and both, respectively. Heat-denatured soluble erythropoietin receptor did not affect the proliferation of BRECs induced by vitreous samples from four patients with proliferative diabetic retinopathy (Fig. 2D).

EXPRESSION OF ERYTHROPOIETIN AND VEGF IN ISCHEMIC RETINAS OF MICE

Retinal levels of erythropoietin messenger RNA (mRNA) in mice increased dramatically when they

Table 2. The Proportion of Patients with Proliferative Diabetic Retinopathy in Each Quartile of the Vitreous Levels of Erythropoietin and Vascular Endothelial Growth Factor (VEGF).

Variable	Patients with Proliferative Diabetic Retinopathy*
	no. (%)
Percentile of erythropoietin levels	
0 to 25th	0
26th to 50th	9 (25.0)
51st to 75th	29 (80.6)
76th to 100th	35 (97.2)
Percentile of VEGF levels	
0 to 25th	2 (5.6)
26th to 50th	7 (19.4)
51st to 75th	31 (86.1)
76th to 100th	33 (91.7)

* There were 144 patients (36 in each quartile).

Table 3. Logistic-Regression Analyses Showing the Association of Erythropoietin and VEGF with Proliferative Diabetic Retinopathy.

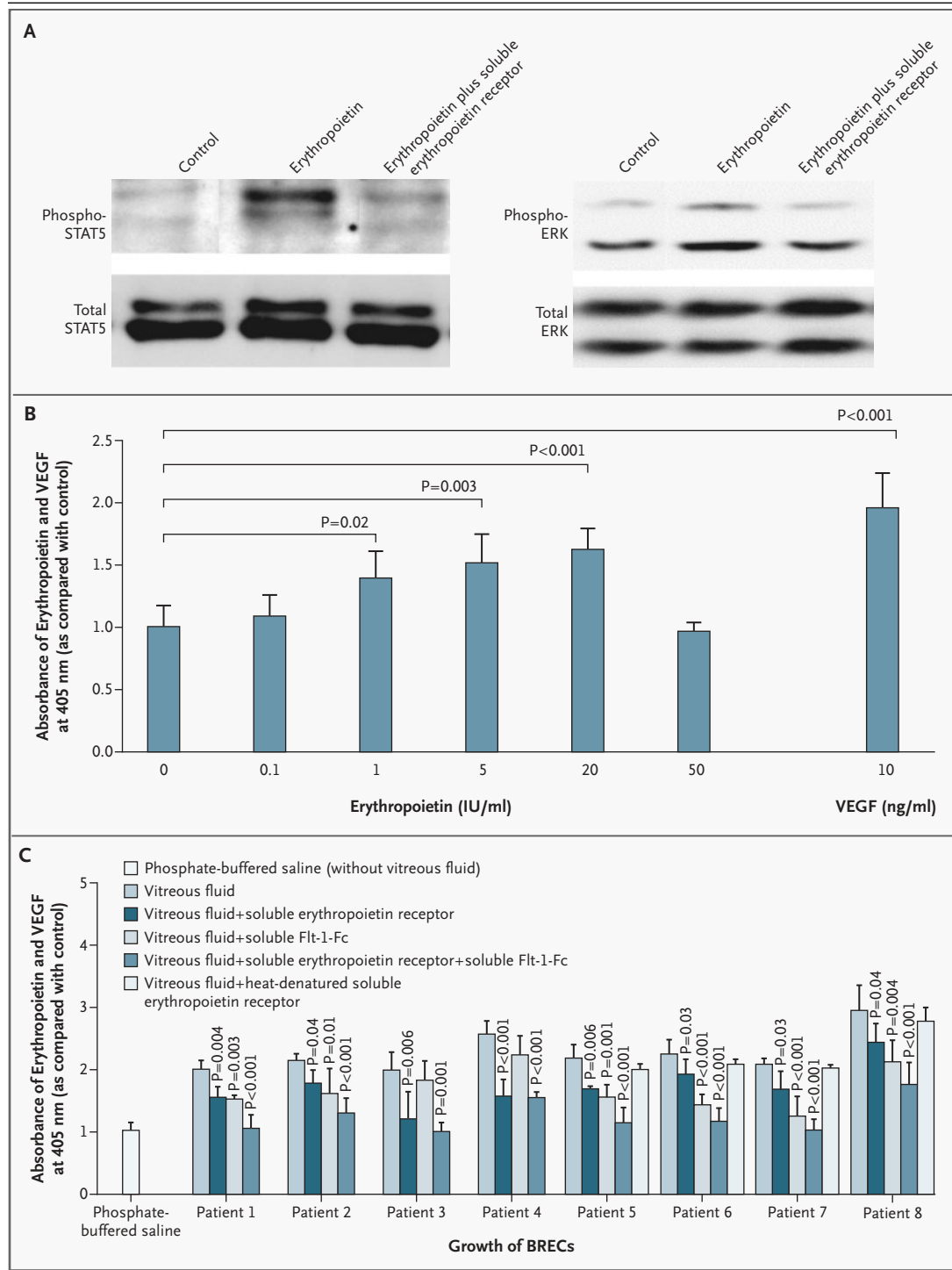
Analysis	Odds Ratio (95% CI)*	P Value
Univariate logistic regression		
Erythropoietin	49.78 (14.76–167.89)	<0.001
VEGF	13.04 (6.22–27.35)	<0.001
Multivariate logistic regression (4 covariates)		
Erythropoietin	27.51 (5.95–127.12)	<0.001
VEGF	4.51 (1.88–10.82)	0.001
Age (per yr)	1.03 (0.97–1.09)	0.39
Sex (M vs. F)	0.77 (0.16–3.69)	0.74
Multivariate logistic regression (2 covariates)		
Erythropoietin	22.46 (5.80–87.01)	<0.001
VEGF	4.24 (1.84–9.76)	0.001

* For erythropoietin and VEGF, the odds ratio pertains to an increase of 1 SD in a base-10 log-transformed scale. CI denotes confidence interval.

were 17 days old, then decreased somewhat on day 19. The levels of VEGF mRNA were also increased on day 17 and paralleled the changes to erythropoietin transcript levels (Fig. 3A).

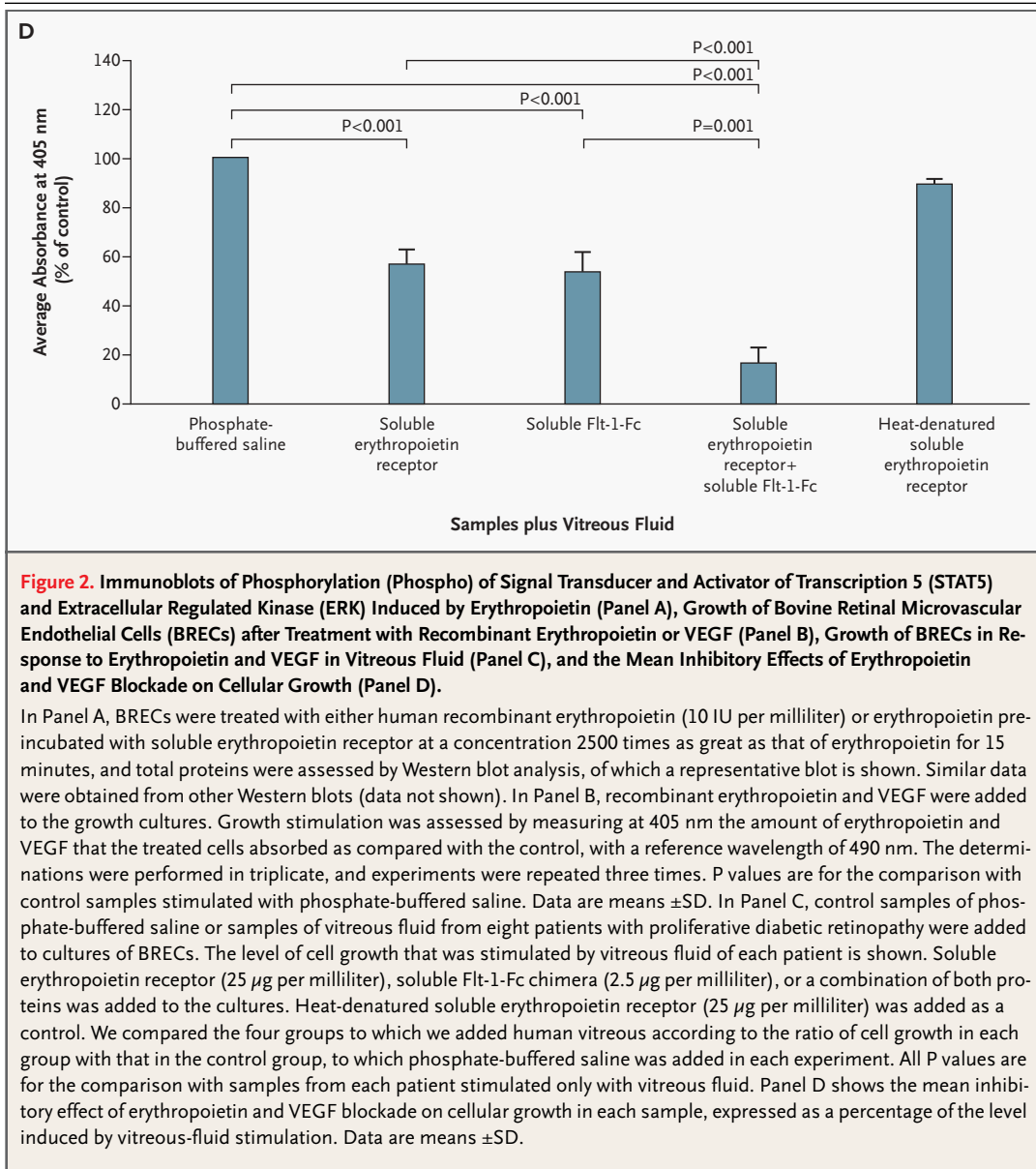
SOLUBLE ERYTHROPOIETIN RECEPTOR AND SOLUBLE Flt-1-Fc IN AN IN VIVO MODEL OF ISCHEMIA-INDUCED RETINAL NEOVASCULARIZATION

Intraocular injections of soluble erythropoietin receptor reduced histologically evident retinal neovascularization in 19-day-old mice in a dose-depen-



dent manner as compared with equivalent injections of human IgG or heat-denatured soluble erythropoietin receptor. The mean percentage inhibition, as compared with values in the contralateral eye injected with IgG, was 65, 59, and 55 percent after

injection of 25, 62.5, and 250 ng of soluble erythropoietin receptor, respectively. The observed maximum inhibition of retinal neovascularization by soluble erythropoietin receptor treatment was found to be at least as great as that with soluble Flt-1-Fc

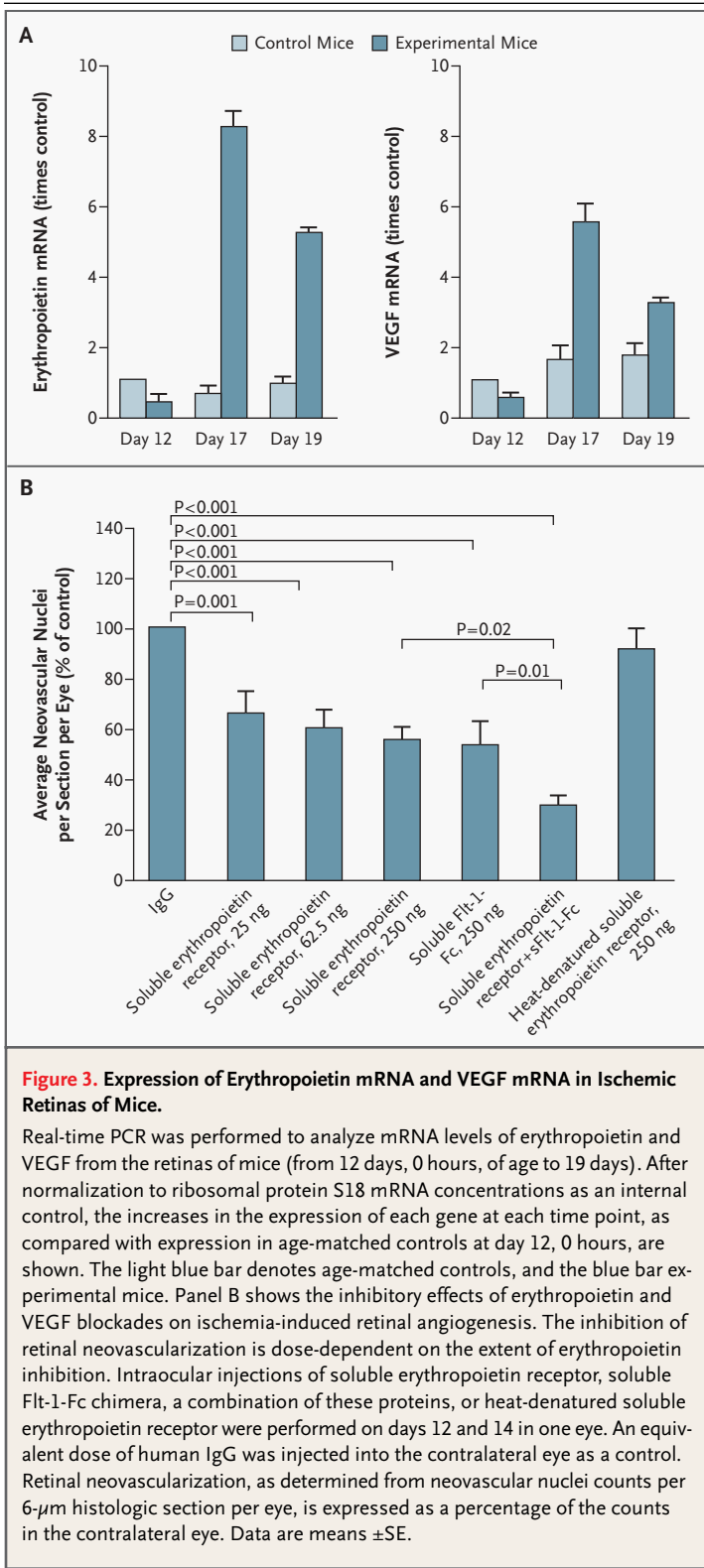


(52 percent), with no statistically significant difference ($P=0.81$). The mean percentage inhibition of neovascularization was 30 percent when a combination of soluble erythropoietin receptor and soluble Flt-1-Fc was injected (Fig. 3B).

DISCUSSION

The present study indicates that the level of erythropoietin in the vitreous fluid of patients with proliferative diabetic retinopathy is strikingly higher than the level in nondiabetic patients. With the use

of multivariate logistic-regression analyses, we observed that erythropoietin and VEGF were each independently associated with proliferative diabetic retinopathy. Indeed, erythropoietin was more strongly associated with proliferative diabetic retinopathy than was VEGF. No significant correlation was observed between the vitreous and plasma levels of erythropoietin, suggesting that increased erythropoietin levels in the vitreous fluid are probably due to increased local production in the retina, as we have shown in the murine model of ischemia-induced retinal neovascularization.



The presence of erythropoietin in the vitreous is probably not due to the breakdown of the blood-retinal barrier. However, we cannot rule out the possibility of an intraocular mechanism for controlling levels of erythropoietin, much like the mechanism that increases ascorbate in the aqueous humor (i.e., saturation kinetics).^{23,24} Ascorbate protects ocular tissues such as the lens against free radicals produced mainly by ultraviolet radiation.

Although we observed dramatic and concomitant up-regulation of both VEGF and erythropoietin in an experimental murine model of ischemic retinas, we observed only a weak correlation between the vitreous levels of erythropoietin and VEGF in patients with proliferative diabetic retinopathy. It is well documented that erythropoietin is up-regulated in situations of hypoxia by a molecular mechanism similar to that of VEGF, including transcriptional activation by hypoxia-inducible factor²⁵ and increased mRNA stability.²⁶ We also observed a slight increase in the vitreous erythropoietin level in patients with inflammatory eye diseases. Although erythropoietin, like VEGF, is an ischemia-induced local retinal factor, the present data suggest that a stimulant other than ischemia, such as high glucose levels, oxidative stress, intraocular inflammation, or the presence of other cytokines, may also differentially affect erythropoietin expression.

Other evidence also supports the concept that erythropoietin is involved in proliferative diabetic retinopathy. Erythropoietin stimulates proliferation of BRECs in a dose-dependent way. However, erythropoietin may have a biphasic effect on endothelial proliferation much as VEGF does.²⁷⁻²⁹ Erythropoietin in the vitreous fluid of patients with proliferative diabetic retinopathy is bioactive and stimulates proliferation of BRECs. The blockade of erythropoietin inhibited the stimulation of cell growth in vitro as efficiently as did VEGF, suggesting that erythropoietin might have angiogenic potency equivalent to that of VEGF in patients with proliferative diabetic retinopathy. The combination of soluble erythropoietin receptor and soluble Flt-1-Fc reduced the proliferation of BRECs, though the inhibition with both proteins was still incomplete when samples from Patients 2, 4, and 8 were used. These in vitro results suggest that although VEGF and erythropoietin seem to have major roles in the pathogenesis of proliferative diabetic retinopathy, other growth factors may also be

involved. Our results with the *in vivo* model are consistent with this hypothesis.

Several reports show the efficacy of treatment with erythropoietin for various diseases. Correcting anemia with erythropoietin therapy may slow the progression of renal failure³⁰ and may reduce the progression of tumors.³¹ Erythropoietin treatment can counter neural damage in patients who have had strokes.³² Small case studies of patients with diabetic nephropathy indicate that treating anemia with erythropoietin improves diabetic retinopathy.^{33,34} However, patients in those studies were also being aggressively treated for other coexisting conditions that can affect retinopathy (e.g., hypertension, hyperlipidemia, and azotemia). Furthermore, both of those reports lacked comparison groups of untreated patients.^{33,34} In addition, erythropoietin is used to treat anemia, and this may confound observations, since anemia is a risk factor for diabetic retinopathy.³⁵

Erythropoietin blockade is likely to be beneficial for the treatment of proliferative diabetic retinopathy. However, erythropoietin blockade may be haz-

ardous for retinal diseases that involve apoptosis of retinal photoreceptors.³⁶ In fact, this strategy might worsen diabetic neuropathy if administered indiscriminately,³⁷ because erythropoietin is a survival factor for retinal photoreceptors^{38,39} and acts as a neurologic protection factor in diabetic neuropathy.^{37,40} Conversely, the possibility that erythropoietin might have a beneficial effect on neuronal damage may be counterbalanced by risk for patients who also have retinal vasoproliferative diseases. Local as opposed to systemic therapy might potentially overcome these problems. Further studies, including the analysis of neuronal side effects, will be necessary to determine whether erythropoietin blockade would work as an approach to the treatment of proliferative diabetic retinopathy.

Supported by grants-in-aid for scientific research from the Japanese Ministry of Education, Science, and Culture and Ministry of Health and Welfare.

We are indebted to Dr. Tetsuji Yamashita (Mitsubishi Kagaku Bioclinical Laboratory) for his technical advice and assistance with ELISA and radioimmunoassay techniques and to Dr. Lois E.H. Smith (Department of Ophthalmology, Children's Hospital and Harvard Medical School) and Dr. Nobuyuki Takakura (Cancer Research Center, Kanazawa University) for their helpful discussion.

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