Rat strain-dependent susceptibility to ischemia-induced retinopathy associated with retinal vascular endothelial growth factor regulation

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Abstract

Vascular endothelial growth factor (VEGF) is a potent inflammation, vascular permeability, and angiogenic factor. Variations of the *VEGF* gene are implicated in the pathogenesis of diabetic retinopathy. Previous studies have shown that Brown Norway (BN) rats have higher retinal VEGF levels and more severe retinal vascular leakage than Sprague-Dawley (SD) rats in response to ischemia and diabetes. To investigate the molecular mechanism of vascular leakage in this animal model, F2 progeny were generated by crossbreeding BN and SD rats. Neonatal rats were exposed to hyperoxia to induce oxygen-induced retinopathy (OIR) models. The F2 rats in response to ischemia have shown a linear distribution of retinal VEGF levels, which is significantly and positively correlated to retinal vascular leakage. We identified a single nucleotide polymorphism (SNP) at upstream stimulating factor-binding site in the VEGF promoter region between BN and SD rats. No differences were found in retinal vascular permeability or VEGF levels between F2 rats with BN, SD, and BN/SD alleles of VEGF SNP. The increased retinal VEGF levels are correlated to ischemia-induced retinal vascular leakage in the OIR rat model. The VEGF mRNA and promoter are not responsible for increased retinal VEGF level and vascular permeability. The up-regulation of VEGF expression activated by a yet to be identified upstream factor or mediator affecting VEGF stability may be associated with a high susceptibility to retinal vascular leakage in BN rats.

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Introduction

Retinal vascular leakage or breakdown of the blood–retinal barrier is a common pathogenic feature of diabetic retinopathy (DR) in diabetic patients and streptozotocin (STZ)-induced diabetic animal models (Gardner *et al.* 2002, Hikichi *et al.* 2002). The increased retinal vascular permeability can result in diabetic macular edema (DME) and subsequently retinal neovascularization (NV; Polkinghorne *et al.* 1992, Aiello *et al.* 1997). Retinal NV ultimately causes severe vitreous cavity hemorrhage and/or retinal detachment, resulting in vision loss.

Vascular endothelial growth factor (VEGF) is a potent mediator of vascular permeability and angiogenesis in a variety of human pathological situations. The increased VEGF levels are responsible for the retinal vascular leakage or retinal vascular hyperpermeability, and retinal NV (Aiello *et al.* 1998, Antonetti *et al.* 1999, Gardner *et al.* 2000, Ishida *et al.* 2003). A number of clinical and animal studies have shown that VEGF plays a pivotal role in normal and pathological angiogenesis, for example, the development of DR (Chiarelli *et al.*

2000, Caldwell *et al.* 2003). The up-regulated expression of retinal VEGF and its receptors correlates to retinal vascular leakage in oxygen-induced retinopathy (OIR) and STZ-induced diabetic rats (Adamis *et al.* 1994, Pierce *et al.* 1995). The inhibition of VEGF and VEGF receptors has been found to prevent retinal NV in diabetic and OIR animal models (Gilbert *et al.* 1998, Qaum *et al.* 2001).

Genetic variations in the VEGF mRNA, VEGF promoter, and VEGF receptors are involved in diabetic microvascular complications (Awata *et al.* 2002, Ikeda 2003, Yang *et al.* 2003, Ray *et al.* 2004). The VEGF -460C polymorphism reversed from the transcriptional start site in the promoter region is associated with proliferative DR and a positive independent predictive factor for the development of proliferative DR (Ray *et al.* 2004). The C(-634)G polymorphism reversed from the translational start site in the 5'UTR of the *VEGF* gene is strongly associated with an increased risk of retinopathy (Awata *et al.* 2002). VEGF variants with an intact receptor binding interface inhibit VEGF-stimulated receptor phosphorylation and proliferation of human endothelial cells (Siemeister *et al.* 1998).

Our recent studies have found that there is a strain difference in susceptibility to retinal vascular leakage between Brown Norway (BN) and Sprague–Dawley (SD) rats in response to ischemia and diabetes (Gao *et al.* 2001, 2002, Zhang *et al.* 2005). Under the same stress conditions, BN rats have higher levels of VEGF mRNA and protein with lower protein levels of pigment epithelium-derived factor (PEDF) in the retina and more severe retinal vascular leakage and NV than SD rats. However, the mRNA level of PEDF was not changed in OIR animal models. PEDF may be one of the factors affecting VEGF expression. We hypothesize that VEGF, a potent vascular permeability factor, is a candidate gene responsible for susceptibility to retinal vascular leakage in the OIR-BN model. In this study, we searched the polymorphisms of *VEGF* gene between BN and SD rats and compared the expression levels of VEGF and vascular permeability in the retina of BN, SD, and F2 progeny of BN and SD crossbreeding.

Materials and methods

Animals

BN and SD rats were purchased from Charles River Laboratories (Wilmington, MA, USA). BN males and SD females were crossbred to produce the F1 generation rats (BN/SD) with BN and SD strain rat background. To generate F2 animals, F1 BN/SD males and females were intercross-bred. Care, use, and treatment of all animals in this study were in strict agreement with the guidelines for the Care and Use of Laboratory Animals by the University of Oklahoma.

Oxygen-induced retinopathy model

The induction of OIR models was performed as described by Smith *et al.* with some modifications as in our previous method (Zhang *et al.* 2001). Briefly, newborn BN, SD, and BN/SD F2 rats were randomly assigned to experimental and control groups. On postnatal day 7 (P7), rats were exposed to hyperoxia (75% O₂) for 5 days (P7–P12) and then returned to room air for 4 days (P16) to induce OIR-rats because our previous studies have shown that all oxygen-treated rats on postnatal day 16 (P16) developed severe retinal vascular leakage (Zhang *et al.* 2005). Control rats were kept in constant normoxia conditions.

Evaluation of retinal vascular permeability

Retinal vascular permeability was quantified by measuring albumin leakage from blood vessels into the retina following the previous methods (Bhatia *et al.* 1998, Xu *et al.* 2001, Ishida *et al.* 2003) with modifications. The rat was anesthetized by inhalation

with isoflurane. The fluorescein isothiocyanate (FITC)-labeled albumin in phosphate buffer solution (PBS; 10 mg/kg body weight, Sigma) was injected into the femoral vein under microscopic inspection. After the injection, the rats were kept in room temperature for 3 h. Then the rat chest cavity was opened and blood was collected through the right atrium. To clear the remaining intravascular FITC-albumin, each rat was perfused via the left ventricle with 50 ml of 1×PBS (pH 7.4), which was pre-warmed to 37 °C to prevent vasoconstriction. Immediately after the perfusion, the eyes were enucleated and the retinas were carefully dissected under an operating microscope. The retina was homogenized in 200 µl PBS containing the proteinase inhibitor cocktail (Roche Diagnosis). The FITC-albumin leaked into the retina was extracted by sonication and centrifugation. The fluorescence intensity (FI) of FITC-albumin from supernatant and serum was measured at excitation wave 485 nm/emission wave 530 nm in Wallac Victor³ multilabel counter (Perkin–Elmer Life and Analytical Science, Turku, Finland). Retinal protein levels in the same wells were measured in the multilabel counter at A280 and A490. The leaked FITC-albumin levels in the retina were normalized by serum fluorescence intensity and retinal protein level. Retinal vascular permeability was calculated as the following formula: Retinal FITC-albumin (µg)/serum FITC-albumin concentration (µg/µl)/retinal protein level (A280)/circulation time. The results of retinal vascular permeability were expressed as retinal FI/serum FI/retinal A280.

ELISA for retinal VEGF levels

The ELISA kits for rat VEGF were purchased from R&D Systems, Inc (Minneapolis, MN, USA). The retinal samples as mentioned above were used for the measurement of VEGF levels. ELISA for VEGF was performed according to the manufacturer's instructions. The retinal VEGF levels were normalized by retinal protein levels at A280.

Genomic DNA purification

Rat genomic DNA was extracted using the salt precipitation method (Klett *et al.* 2004). Rat tail (about 0.5 cm) was digested in 500 µl lysis buffer (50 mM Tris–HCl (pH 8.0), 100 mM EDTA, 125 mM NaCl, 1% SDS, 200 µg Proteinase K) at 55 °C with rocking overnight. The saturated NaCl (200 µl) was added into digested solution with vigorous shaking for at least 60 s. Mixed solution was spun down at maximal speed in top microcentrifuge for 20 min. The supernatant was transferred to a new tube with an equal volume 100% ethanol, mixed by inversion and spun

down. The DNA pellet was washed in 70% ethanol and suspended in 1000 μ l Tris–EDTA buffer. DNA concentration was quantified in Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA).

DNA sequencing

The *VEGF* gene DNA fragments from BN, SD, and F2 BN/SD rats were amplified by PCR using rat genomic DNA, Master PCR kits (Roche Diagnosis), and primer sets as shown in Table 1. The PCR products were purified using PCR product purification kits (Roche Diagnosis). The purified DNAs were amplified using internal primers and sequencing PCR kit and were sequenced on the ABI 3730 capillary DNA sequencer by the Oklahoma Medical Research Foundation Sequencing Core.

Analysis of single nucleotide polymorphism (SNP)

The SNP of the upstream stimulating factor (USF)-binding site in the VEGF promoter region between BN and SD rats was analyzed using Kaufman's method with some modifications (Kaufman *et al.* 2006) in the following steps. The polymorphism of USF-binding site and primer design are shown in Fig. 4A and B.

Step 1: standard PCR amplification

The DNA fragments including USF SNP of BN, SD, and BN/SD F2 rats were amplified by PCR in 5 μ l reaction solution which contains 5 ng rat genomic DNA, 150 nM USF-F and USF-R primers, and 1 $\times$ Master PCR buffer. The PCR conditions were cycled as follows: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min for 35 cycles.

Step 2: PCRs cleanup

PCR products were treated with shrimp alkaline phosphatase (SAP) to remove excess dNTPs. SAP (0.2 Unit; Roche Diagnosis) in 5 μ l of 50 mM Tris–HCl buffer (pH 7.4) was added into the PCR product solution. The reaction solution was incubated at 37 °C for 30 min and denatured at 70 °C for 15 min to inactive the enzyme.

Step 3: primer extension

For primer extension reaction, dATP-missed dNTP was used in the PCR to stop primer extension at adenine base. USF Extension-R primer was synthesized and labeled with fluorescence IR Dye 700 by Li-Cor, Inc (Lincoln, NE, USA). The primer extension was performed in 5 μ l PCR buffer containing 2 μ l SAP-treated PCR products, 0.1 pmol Dye 700-labeled extension primer, 250 nM dCTP, dGTP, and dTTP, and 1 $\times$ PCR buffer. Primer extension PCR was 94 °C, 2 min for denaturing, and 10 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s.

Step 4: fragmentation analysis

The PCR products of primer extension were loaded on 15% polyacrylamide gels and analyzed on a Li-Cor 4200 automated DNA sequencing system (Li-Cor) following the manufacturer's recommendations. The genotypes of USF SNP in F2 progeny were analyzed by sequencing system software as shown in Fig. 5C.

Statistical analysis

Data are shown as mean $\pm$ s.d. Student's *t*-test was used to determine the statistical significance of differences

Table 1 Primers of vascular endothelial growth factor gene for PCR and sequencing

Primer name	Position	Sequences 5' to 3'
VEGF promoter 1290-F	–1290	AGT TCC CTG GCA ACA TCT GG
VEGF promoter 1290-R	–1271	CCA GAT GTT GCC AGG GAA CT
VEGF promoter 2790-F	–2790	TGA CCT GCT TTT GGG GGT GA
VEGF promoter 1780-F	–1780	CAC TAG GGG GCG CTC GGC CA
VEGF promoter 1761-R	–1761	GCG CGA GCG CCC CCT AGT GA
VEGF promoter 2280-F	–2280	ACC GCT GTT ACC GGT GAG AA
VEGF promoter 2410-R	–2410	GCT GAT GAG TCC GTT GAA TA
VEGF promoter 100-F	–100	ATC ACG GCT GTC TTG GGA TA
VEGF promoter 580-F	–580	GGT GAC TCA GGG CAG CTA T
VEGF promoter 640-R	–640	CAA TGG AGC AAT GTT ATG AGG A
VEGF promoter USF-F	–1285	GAG CCA CAC AGG CTA AGT GTA
VEGF promoter USF-R	–1151	CTA GCC TAG GCT CAC AGT TCA
VEGF 33R	33	CCC AAG CCT CTG CGC TTC T
VEGF SNP1-F	117	GGT GAA GTT CAT GGA CGT CTA
VEGF SNP1-R	289	TCT GCA TAG TGACGTTGCTCT

between the groups of animals. Significance was set at $P < 0.05$. The association of retinal VEGF levels with retinal vascular permeability was tested using ANOVA linear regression analysis program.

Results

Increased retinal vascular permeability and VEGF protein levels in BN rats in response to ischemia

Our previous studies have shown that retinal permeability is significantly increased, with a peak at P16 in OIR-BN rats, when compared with the age-matched normal control BN and SD rats. The increase in permeability is greater in OIR-BN rats than in OIR-SD rats (Zhang *et al.* 2005). To determine whether this susceptibility will be inherited to offspring generations, F2 progeny with BN and SD background was generated. Before crossbreeding of inbred BN rats and outbred SD rats, the retinal vascular permeability was assayed at P16 in control, OIR-BN and SD rats to confirm that the breeding BN rats are susceptible to retinal vascular leakage in response to ischemia. There was no difference in the basal levels of retinal vascular permeability between normal control SD and BN rats. In OIR-SD rats, retinal vascular permeability was 1.74-fold higher than that in the control SD rats ($P < 0.05$, $n = 4$; Fig. 1A). However, retinal vascular permeability in OIR-BN rats was 4.97-fold higher than that in the age-matched control BN rats ($P < 0.001$, $n = 4$) and 2.85-fold higher than that in OIR-SD rats ($P < 0.001$, $n = 4$; Fig. 1A). The results suggest that BN rats develop more severe vascular leakage than SD rats under the same ischemia stress.

The basal levels of retinal VEGF protein were similar in normal BN and SD rats. In OIR-SD rats, retinal VEGF protein levels had no significant increase over those in normal control SD rats (Fig. 1B). However, in OIR-BN rats, retinal VEGF levels were approximately tenfold higher than those in normal control BN and OIR-SD rats ($P < 0.001$, $n = 4$; Fig. 1B).

Distribution and association of retinal VEGF protein levels with retinal vascular permeability in F2 progeny BN/SD rats in response to ischemia

To determine if retinal VEGF levels are correlated to retinal vascular permeability, the retinal vascular permeability and VEGF contents at P16 were quantified in 97 F2 SD/BN rats exposed to 75% oxygen for 5 days and to room air for 4 days. The retinal VEGF contents in oxygen-treated F2 rats had a linear distribution in the range of 22–666 pg/mg protein (Fig. 2A). The retinal VEGF levels were significantly positively correlated to retinal vascular permeability in F2 SD/BN rats in

Figure 1 Retinal vascular permeability (A) and VEGF levels (B) in OIR-BN and -SD rats. (A) The retinal vascular permeability was performed in normal control and OIR-SD and -BN rats at P16 and expressed as ng FITC-albumin/mg retinal protein (mean $\pm$ s.d., $n = 4$). (B) The retinal VEGF levels were measured by ELISA, normalized by retinal protein, and expressed as pg/mg protein (mean $\pm$ s.d., $n = 4$). Values significantly higher than the age-matched normal control are indicated by * $P < 0.05$ and † $P < 0.001$.

response to ischemia ($n = 97$, $R = 0.7575$, $P < 0.001$; Fig. 2B). The F2 rats with extreme high retinal vascular permeability had approximately fivefold higher retinal VEGF contents than in those with extreme low retinal vascular permeability (Fig. 3A and B).

Polymorphism of the VEGF promoter region between BN and SD rat strains

Since retinal VEGF contents were significantly associated with retinal vascular leakage in oxygen-treated F2 BN/SD rats, it is proposed that *VEGF* be one of the candidate genes responsible for susceptibility to vascular leakage in OIR-BN rats. First, we searched the rat sequence database (<http://rgd.mcw.edu/tool-entry.shtml> and <http://www.ncbi.nlm.nih.gov/>) to determine if there is any polymorphism of the *VEGF* gene between SD and BN rat strains. There are two

Figure 2 Distribution and correlation of retinal VEGF levels with retinal vascular permeability in OIR F2 BN/SD rats. (A) The OIR F2 rats show a continuous distribution of retinal VEGF levels. (B) Retinal vascular permeability (X-axis) was plotted against retinal VEGF levels (Y-axis). The permeability is expressed as FITC-albumin/mg retinal protein. The correlation between VEGF levels and permeability was analyzed by linear regression ($n=97$, $r=0.7575$, $P<0.001$).

SNPs (*rs8152485* and *rs8148303*) of the *VEGF* gene between BN and SD rat strains according to the existing information in the database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp>). The code of VEGF SNP (*rs8148303*, A/G) does not change the amino acid residue. Another VEGF SNP (*rs8152485*) is A/G (BN/SD) at 209 of the VEGF mRNA sequence from transcription initiation site, which causes amino acid substitution, tyrosine (Tyr)/cysteine (Cys) (BN/SD) at position 70 of the peptide sequence. Different from the GenBank information, we did not detect any polymorphism of the entire coding region of the VEGF mRNA between breeding BN and SD rats by amplifying and sequencing the VEGF cDNA from BN and SD rats.

In order to determine whether there is any polymorphism in the rat VEGF promoter region, a 2.3 kb fragment of the VEGF promoter was amplified by PCR from the BN and SD rat genomic DNA and sequenced

Figure 3 Retinal VEGF contents in OIR F2 BN/SD rats with extreme low and high retinal vascular permeability. To verify that high retinal VEGF contents are related to hyperpermeability, the VEGF contents were compared between 19 rats with extreme low permeability and 29 rats with extreme high permeability. The results show that the rats with extreme high permeability have five times higher VEGF contents than rats with extreme low permeability. $**P<0.001$ extreme low vs extreme high groups.

(Table 1). One SNP (G/T) was found at -1234 in the VEGF promoter region between BN and SD rat strains. When compared with the SD rat sequence, BN rats lack the binding site of USF in the VEGF promoter region (Fig. 4A).

No differences in retinal VEGF contents and retinal vascular permeability between the BN, SD, and BN/SD alleles of the VEGF promoter SNP in F2 progeny

To determine if the genetic variation of VEGF USF-binding site polymorphism is associated with the susceptibility to vascular leakage in the BN rat strain, the retinal VEGF contents and vascular permeability were measured in F2 BN/SD rats in response to ischemia. The VEGF USF-binding site SNP was genotyped by primer extension method using genomic

Figure 4 The polymorphisms in the VEGF promoter from SD and BN rats. (A and B) Partial sequences of the VEGF promoter in BN and SD rats respectively. The nucleotide polymorphisms are underlined at -1234 of the VEGF promoter region. The sequence (GGAGAATGGGA, underlined) is the putative binding site of USF. The variation of BN strain results in the loss of the USF-binding site. (C) The sequence and primer design for genotyping of the USF-binding site SNP. The primers, USF-F and USF-R underlined, were used to amplify 119 bp VEGF promoter fragments containing the USF-binding site SNP (G/T, italic). Extension-R underlined and italic was used for primer extension amplification. The primer extension PCR solution does not contain dATP, therefore primer extension stopped at A base of the complement strand. In the SD strain, the primer was extended for eight bases (TTCTCCCT), but in the BN strain, the primer can be extended for only four bases (TTCT) because the nucleotide base TTCT is adenine (A) in BN strain. (D) PAGE. The bottom bands represent original primer size (20 bases). The middle bands are the BN allele (24 bases) and the top bands are the SD allele (28 bases). Lane 23 is outbred SD, lane 22 is inbred BN. Lanes 9, 11, 12, and 20 show BN allele in F2 rats. Lanes 3, 6, 7, 14, 19 and 21 are SD alleles in F2 rats. Lanes 1, 2, 4, 5, 8, 10, 13, and 15-18 present heterozygous BN/SD allele in F2 rats.

DNA from oxygen-treated F2 rats. The retinal vascular permeability showed no significant differences between the heterozygous BN/SD, homozygous BN, and SD alleles of VEGF USF SNP (Fig. 5A). The distributions of retinal VEGF contents between BN, SD, and BN/SD

allele rats were 22–666 ($n=28$), 28–534 ($n=17$), and 25–601 ($n=39$) pg/ μ g protein respectively (Fig. 5B). There is no significant difference in retinal VEGF levels between the BN, SD, and BN/SD alleles of the VEGF SNP.

Figure 5 Retinal VEGF levels and vascular permeability in BN, SD, and BN/SD alleles of the VEGF USF SNP. (A) The distribution of retinal vascular permeability in F2 rats with homozygous BN, homozygous SD, and heterozygous BN/SD alleles of VEGF USF SNP respectively. ▲, BN allele; ▽, SD allele; ◆, BN/SD allele. Horizontal lines represent the average values. The retinal vascular permeability of F2 rats in homozygous BN, homozygous SD, and heterozygous BN/SD alleles were 69.67 ± 25.93 , 56.68 ± 22.71 , and 53.25 ± 24.21 respectively. There is no difference of retinal vascular permeability between BN, SD, and BN/SD alleles. (B) The distribution of retinal VEGF levels in F2 rats with homozygous BN, homozygous SD, and heterozygous BN/SD alleles of the VEGF USF SNP respectively. The retinal VEGF levels of F2 rats in the BN, SD, and BN/SD alleles were 242.18 ± 167.88 , 208.40 ± 162.48 , and 220.54 ± 160.0 respectively. All of the three alleles of F2 rats have the similar distribution in retinal vascular permeability and VEGF contents.

Discussion

DR is a chronic and progressive retinal capillary disorder. In the early stage of diabetes, retinal vascular permeability can increase even before the appearance of clinical retinopathy (Sheth 1999, Russ *et al.* 2001). Retinal vascular leakage and thickening of the retina lead to DME. In the late stage of DR, over-proliferation of

capillary endothelial cells results in retinal NV, abnormal formation of new vessels in the retina and in the vitreous, leading to proliferative DR (PDR; Shiels *et al.* 1998, Stitt *et al.* 2000). The abnormal angiogenesis ultimately causes severe vitreous cavity bleeding and/or retinal detachment, resulting in severe vision loss. Diabetic patients have variable susceptibilities to retinopathy. The genetic variations are risk factors for DR and other vascular complications in diabetic patients, and different animal strains (Gao *et al.* 2001, 2002, Kitzmann *et al.* 2002, Chan *et al.* 2005, Zhang *et al.* 2005). Our recent studies have shown that BN rats are genetically more susceptible to retinal vascular leakage induced by ischemia than the SD rats (Gao *et al.* 2001, 2002, Zhang *et al.* 2005). In this study, we confirmed the previous results that OIR-BN rats at P16 have retinal vascular permeability approximately five times higher than that in age-matched normal control rats and approximately three times higher than the OIR-SD rats.

The molecular mechanisms for the different susceptibility to vascular leakage in response to ischemia and diabetes are not clear. Multiple genes, such as *VEGF*, *PEDF*, aldose reductase (AR), nitric oxide synthase (NOS2A and NOS3), protein kinase C- β (PKC- β), etc, are implicated in the pathogenesis of DR. Alterations of these growth factors and their receptors in diabetes have been identified in both experimental and clinical DR (Casey & Li 1997, Gerhardinger *et al.* 2001). VEGF is a potent mediator of vascular permeability and angiogenesis, and a potent mitogen with a unique specificity for endothelial cells in a variety of human pathological conditions. The increased VEGF levels are responsible for the retinal vascular leakage or retinal vascular hyperpermeability, and retinal NV. A number of clinical and animal studies have shown that VEGF plays a pivotal role in the development of DR (Chiarelli *et al.* 2000, Caldwell *et al.* 2003). The up-regulated expression of retinal VEGF and its receptors correlate to high retinal vascular permeability (RVP) in OIR and STZ-induced diabetic rats (Adamis *et al.* 1994, Aiello *et al.* 1994, Pierce *et al.* 1995). It has been known that VEGF expression is stimulated by hypoxia (Gao *et al.* 2002, Zhang *et al.* 2005), high glucose levels (Iglesias-de la Cruz *et al.* 2002, Omori *et al.* 2004), advanced glycosylation end products (Yamagishi *et al.* 2002, Yokoi *et al.* 2005), IGF-I (Hellstrom *et al.* 2001), and angiotensin II (Funatsu *et al.* 2002). Inhibition of VEGF and VEGF receptors has been shown to prevent retinal NV in diabetic and OIR animal models (Gilbert *et al.* 1998, Qaum *et al.* 2001). The present study demonstrated that OIR-BN rats have retinal VEGF levels more than ten times higher than those in OIR-SD rats. OIR F2 BN/SD strain rats have a continuous distribution of retinal VEGF levels, which is positively correlated to retinal vascular permeability. The F2 rats with extreme high retinal vascular permeability have retinal VEGF

levels five times higher than those with extreme low retinal vascular permeability.

The susceptibility for vascular leakage may be a polygenic mode of inheritance. *VEGF* would be a strong candidate gene responsible for susceptibility to vascular leakage. It has been reported that the C(−634)G polymorphism in the 5'UTR of the *VEGF* gene is strongly associated with an increased risk of retinopathy (Awata *et al.* 2002). The VEGF −460C polymorphism-based transcriptional start site is associated with proliferative DR and a positive independent predictive factor for the development of proliferative DR (Ray *et al.* 2004). VEGF mutants at Cys51Ser and Cys60Ser have low potency in inducing proliferation in human umbilical vein endothelial cells (Leenders *et al.* 2001). We hypothesize that *VEGF* is a candidate gene responsible for the higher susceptibility to vascular leakage in OIR-BN rats. Two SNPs of the *VEGF* gene between BN and SD rat strains are found in the rat sequence database. One SNP (rs8148303, A/G) does not change peptide residue. Another VEGF SNP (rs8152485, A/G), located at 209 of the VEGF mRNA sequence from transcript initiation site leads to an amino acid change tyrosine (Tyr)/cysteine (Cys) (BN/SD) at 70 of the peptide sequence. However, our studies did not detect these SNP in our breeding BN and SD rat strains. No polymorphism of the VEGF mRNA was detected in the full-length retinal VEGF cDNAs from BN and SD rats.

In order to determine whether VEGF promoter polymorphisms result in elevated retinal VEGF in OIR-BN rats, we amplified and sequenced 2.3 kb DNA fragments of the VEGF promoter from the genomic DNAs of BN and SD rats. One SNP (G/T) was identified at −1234 in the promoter region of VEGF between BN and SD rat strains. The single nucleotide mutation in the VEGF promoter in BN rats results in a loss of the binding site for the USF in the VEGF promoter region. USF, known as major late transcription factor, consists of two related polypeptides of 43 kDa (USF1) and 44 kDa (USF2), and is expressed in every tissue. USF1 regulates several genes related to glucose and lipid metabolism (Casado *et al.* 1999). It has been reported that USF1 is associated with the familial combined hyperlipidemia and affects the complex lipid phenotype (Pajukanta *et al.* 2004, Coon *et al.* 2005). USF proteins induce human *TGF-β1* gene activation via the glucose-response element (Weigert *et al.* 2004). In order to determine whether the genetic variation of the USF transcription factor-binding site in the VEGF promoter region affects VEGF expression in OIR-BN rats, we performed genotyping of USF-binding site SNP allele and measured the retinal VEGF levels and vascular permeability in 97 F2 rats in response to ischemia. Our studies have shown that all of the three rats with the USF SNP allele have similar distribution of

retinal VEGF levels and vascular permeability. These results suggest that the USF SNP *per se* does not affect VEGF expression and that VEGF promoter and mRNA are not responsible for the strain difference in susceptibility to vascular leakage in OIR-BN and OIR-SD rats. The other upstream and intronic transcript factors regulating the VEGF mRNA expression or mediators affecting VEGF protein stability may be associated with vascular leakage in BN rat strain.

In summary, OIR-BN rats have higher retinal VEGF levels and more severe retinal vascular leakage than OIR-SD rats. VEGF levels are positively correlated to vascular leakage in OIR F2 BN/SD rats. No SNP was found in the *VEGF* gene that is associated with different susceptibilities to vascular leakage. These observations suggest that the VEGF promoter and not mRNA is associated with increased retinal VEGF level and vascular permeability; instead, the yet to be identified upstream factors and mediators regulating VEGF expression are responsible for the genetic difference for the retinal vascular leakage. This study lays a solid ground for further elucidation of the genetic basis for the different susceptibilities to DR.

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