Interindividual Heterogeneity of Hypoxic Response of Vascular Endothelial Growth Factor and Intercellular Adhesive Molecule-1 in Monocytes from Diabetic Patients

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INTRODUCTION

Diabetic retinopathy (DR) is one of the leading causes of legal blindness worldwide. The development and progression of DR are related to a variety of factors, such as the age of onset, the duration and type of diabetes, and the level of blood glucose control.[1] However, clinical observations find that considerable variability of the development and progression of DR exists among diabetic individuals. Proliferative DR (PDR) is the advanced stage of DR, and the most distinct finding of PDR is the presence of neovascularization, which is the physiological response of tissues in the condition of hypoxia or ischemia. There are a series of growth factors involved in the neovascularization process.[2] Vascular endothelial growth factor (VEGF) plays a central role in the pathogenesis of DR, while intercellular adhesive molecule-1 (ICAM-1) is a downstream molecular of VEGF in diabetes. In this study, diabetic patients with PDR or without DR were enrolled to test the interindividual heterogeneity in them. The hypoxic response of VEGF and ICAM-1 in monocytes harvested from the patients was investigated.

METHODS

Patients

Thirty-five Type 2 diabetes patients were included prospectively: 17 males and 18 females; mean age 54.4 years old. Patients with hypertension or hyperlipidemia were excluded. All the patients had well-controlled glucose. Patients were divided into two groups. In Group A (n = 16), the patients’ duration of diabetes was <5 years and PDR was present. In Group B (n = 19), the patients had diabetes for over 10 years, and DR was absent. PDR was defined as the presence of neovascularization or fibrovascular membrane. The presence of PDR was determined by an experienced retina specialist Dr. Fang-Tian Dong.

The study was approved by the Institutional Review Board of Peking Union Medical College Hospital. For each patient, a data sheet was completed with the patient’s age, sex, date of the onset of diabetes, duration of diabetes, control of glucose (glucose level, hemoglobin A1c [HbA1c]) in the past several years, and data from an additional examination of the patient’s fundus was also reviewed.

RNA isolation from the monocytes

Mononuclear cells were isolated from peripheral blood by a well-established procedure as previously described.[3] Total RNA was isolated from the monocytes with the TRI Reagent (MRC Inc., USA). Briefly, 1 ml of reagent was added to each sample in 1.5 ml Eppendorf tube. Chloroform...
(200 μl) was added, and the tube was vortexed and centrifuged at 13,150 × g for 10 min. The RNA was precipitated with an equal volume of isopropanol and washed with 80% ethanol. Then, the RNA was air-dried and resuspended in water treated with diethylpyrocarbonate. The optical density of all of the samples was measured at a wavelength of 260 nm.

**Quantitative real-time polymerase chain reaction**

The primers were designed by Primer Express® v.2.0 software (Applied Biosystems, Foster City, CA, USA), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping control gene. The primers of GAPDH-forward (F) and GAPDH-reverse (R), VEGFA-F and VEGFA-R, ICAMI-F and ICAMI-R were 5’-GCAAATTCCATGCGACCCT-3’ and 5’- TCAGCCACCTTGATTTGG-3’, 5’- CGAGGCAGCTTGAGTTAAACG-3’ and 5’- AGATCTGGTTCCCGAAACCCT-3’, 5’- CGGCTCTGAGTCTGAGATATT-3’ and 5’-GGCTTCTGAGTAATTGCTCGT-3’, respectively.

Quantitative real-time polymerase chain reaction (PCR) was carried out in a total volume of 20 μl, with each tube containing 10 μl of 2×SYBR Green Real-time PCR Master Mix (TaKaRa Bio, Japan), 1 μl of template, 0.5 μl of primer forward and reverse (10 μmol/L), respectively. Reactions were run in an MX3000P real-time PCR analyzer (Stratagene, USA) at 95°C for 2 min, then forty cycles of (95°C for 20 s, 56°C for 20 s, and 72°C for 45 s), and then 72°C for 1 min. The tests were repeated three times. To evaluate the basal level of target genes expression, we calculated ΔCT value of the three genes in Group A and Group B as follow:

\[
\Delta CT = CT(\text{target gene}) - CT(\text{GAPDH}).
\]

After the expression of VEGF messenger RNA (mRNA) was normalized, VEGF mRNA expression was normalized to that of GAPDH; the hypoxia/normoxia ratio of expression was calculated as follows:

\[
\Delta \Delta CT = \Delta CT_2 - \Delta CT_1 = (\text{hypoxia VEGF} - \text{hypoxia GAPDH}) - (\text{normoxia VEGF} - \text{normoxia GAPDH}).
\]

Hypoxia/normoxia ratio of expression = \(2^{-\Delta \Delta CT}\).

**Statistical analysis**

The results were expressed as the mean ± standard error (SE). Statistical analysis was calculated using SPSS 15.0 (IBM Corporation, Armonk, NY, USA). The statistical significance of the difference between the ages, duration of diabetes mellitus (DM), HbA1c, level of mRNA in different groups was determined by Mann–Whitney U-test. \(P < 0.05\) was considered statistically significant.

**Results**

**Basic characteristics of patients in the two groups**

In Group A, the mean age was 55.8 ± 6.9 years old, the mean duration of DM was 3.2 ± 0.7 years and the mean level of HbA1c was 7.5% ± 1.9%. In Group B, the mean age was 53.2 ± 8.1 years old, the mean duration of diabetes was 15.4 ± 2.2 years and the mean level of HbA1c was 7.3% ± 1.6%. No statistically significant difference was found between the two groups in age and HbA1c level.

**Levels of vascular endothelial growth factor and intercellular adhesive molecule-1 messenger RNA expression under normoxia and hypoxia in the two groups**

There was no difference in the levels of VEGF and ICAM-1 mRNA expression under normoxia in the two groups (data not shown). In addition, we investigated the VEGF and ICAM-1 mRNA expression under hypoxia in the monocytes harvested from the patients. We found that there was a significant difference in the hypoxia/normoxia ratio of VEGF expression between Group A and Group B (\(P = 0.023\)) [Figure 1]. However, there was no difference in the hypoxia/normoxia ratio of ICAM-1 expression between the two groups (data not shown).

**Discussion**

In this study, we provided evidence to support the hypothesis that the individual heterogeneity of the progression of DR was strongly associated with their ability to induce VEGF gene expression in response to hypoxia. There is a highly significant correlation between increased hypoxia induction of VEGF in those patients with PDR, compared to those patients who do not develop DR, even after many years of diabetes. In Group A, the patients with well-controlled blood glucose developed DR within 5 years, which indicated that those patients were very prone to produce excessive VEGF mRNA. These patients might have a lower hypoxia threshold response. Future work should be focused on what percentage of patients with this response develops PDR.

![Figure 1: Scatter plot of hypoxia/normoxia ratio of VEGF expression in Group A and Group B. Group A: Diabetes duration <5 years and with PDR (n = 16). Group B: Diabetes duration >10 years and without DR (n = 19). \(P = 0.023\) versus Group B. VEGF: Vascular endothelial growth factor; DR: Diabetic retinopathy; PDR: Proliferative diabetic retinopathy.](image-url)
It was proved that an increase in serum VEGF and ICAM-1 levels were associated with an increase in the severity of DR, however, ICAM-1 did not appear as a serum marker for development and progression of DR in this study. It could be explained that only the hypoxic response was tested, which could not cover the all the pathologic changes during the development and progression of DR. Nevertheless, different from ICAM-1, VEGF was shown to be the most important factor involved in several common pathways.

There are a number of immediate conceptual and clinical implications that arise from this study. On the one hand, it provides a potential explanation for the variability in the degree of DR in diabetic patients. Patients identified as high-VEGF responders should be monitored more closely and treated more intensely than low-VEGF responders. It might be suggested for patients identified as high-VEGF responders to receive anti-VEGF therapy at a relatively earlier stage of DR. On the other hand, the demonstration of interindividual variability in the hypoxic induction of VEGF had implications beyond the retina, such as the cardiovascular system and tumors.

This study has several limitations. First, the relatively small number of patients was enrolled in this study. Second, only the levels of VEGF and ICAM-1 from serum monocytes were tested, but not from the aqueous humor or vitreous. A better understanding might be made if samples from serum and vitreous could be tested simultaneously. Finally, only two biomarkers were tested in this study. In future studies, more patients and more DR-related markers will be included.

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Conflicts of interest
There are no conflicts of interest.

References
1. Aiello LP, Gardner TW, King GL, Blankenship G, Cavallerano JD, Ferris FL 3rd, et al. Diabetic retinopathy. Diabetes Care 1998;21:143-56. doi: 10.2337/diacare.21.1.143.
2. Crawford TN, Alfaro DV 3rd, Kerrison JB, Jablon EP. Diabetic retinopathy and angiogenesis. Curr Diabetes Rev 2009;5:8-13. doi: 10.2174/157339909787314149.
3. Marsh S, Nakhoud FM, Skorecki K, Rubin A, Miller BP, Leibu R, et al. Hypoxic induction of vascular endothelial growth factor is markedly decreased in diabetic individuals who do not develop retinopathy. Diabetes Care 2000;23:1375-80. doi: 10.2337/diacare.23.9.1375.
4. Jain A, Saxena S, Khanna VK, Shukla RK, Meyer CH. Status of serum VEGF and ICAM-1 and its association with external limiting membrane and inner segment-outer segment junction disruption in type 2 diabetes mellitus. Mol Vis 2013;19:1760-8.
5. Xie TY, Yan W, Lou J, Chen XY. Effect of ozone on vascular endothelial growth factor (VEGF) and related inflammatory cytokines in rats with diabetic retinopathy. Genet Mol Res 2016;15:1-11. doi: 10.4238/gmr.15027558.