·Clinical Research·

Angiogenesis –related cytokines in serum of proliferative diabetic retinopathy patients before and after vitrectomy

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Abstract

• AIM: To evaluate serum concentrations of angiogenesis-related cytokines in proliferative diabetic retinopathy (PDR) before and after vitrectomy.

• METHODS: Serum samples were collected from 30 PDR patients with varying severity before and after vitrectomy. Serum concentrations of vascular endothelial growth factor (VEGF), pigment epithelium-derived factor (PEDF), interleukin-8 (IL-8) and interferon-inducible protein-10 (IP-10) were determined by enzyme-linked immunosorbent assays(ELISA).

• RESULTS: Serum concentrations of VEGF, PEDF, IL-8 and IP-10 were significantly higher in PDR patients than that in controls, respectively (P<0.05). VEGF concentration decreased significantly in postoperative samples than that in preoperative samples (P<0.05). The concentrations of PEDF, IL-8 and IP-10 did not exhibit significant changes after vitrectomy.

• CONCLUSION: Elevated cytokines levels in serum may be diagnostically useful in PDR. Angiogenesis-related cytokines play important roles in the development of PDR, and would instruct the risk assessment of pathogenetic condition in PDR patients.

• KEYWORDS: proliferative diabetic retinopathy; cytokine; vitrectomy; enzyme-linked immunosorbent assay; angiogenesis

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INTRODUCTION

Systemic microvascular disease is currently the principal pathological change in patients with diabetes mellitus (DM). Diabetic retinopathy (DR), which is a severe complication of DM, is characterized by retinal ischemia, increased vasopermeability, blood retina barrier breakdown and neovasculization in its pathogenesis [1]. Formation of fibrovascular tissue often leads to substantial morbidity and blindness due to vitreous humor and retina hemorrhage, vitreous fibrovascular proliferation and sequent traction retinal detachment [2]. It has been demonstrated that the imbalance between pro- and anti-angiogenesis factors contributed to neovasculization in proliferative diabetic retinopathy (PDR).

Cytokines play quite vital roles in maintaining the normal physiological function of retina. Although the pathogenesis of PDR is still not well understood, the involvements of cytokines, chemokines, inflammatory cells, and angiogenic factors are known to be implicated in the development and progression of PDR. Moreover, some genetic factors may be involved in the pathogenesis of PDR [3,4]. The genetic factors could affect the serum levels of angiogenic factors such as VEGF, intercellular adhesion molecule-1 (ICAM-1) and basic fibroblast growth factor (bFGF) [3-5]. Recently, vitreous and serum cytokines levels have been studied in patients with PDR. Various cytokines, vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) promote neovascular growth, while pigment epithelium-derived factor (PEDF) and interferon-inducible protein-10 (IP-10) exhibit preventive effects against retinal neovascularization. Studies have confirmed that intravitreous injection of bevacizumab, an anti-VEGF antibody, could prevent neovascular growth and diminish intraoperative bleeding in PDR patients. The benefits from bevacizumab would be associated with the inhibition of VEGF expression. PEDF is rich in vitreous and

retina [6,7]. It is regulated by oxygen with a modality different from VEGF and down-regulates VEGF expression. Inflammatory cytokines, including IL-8 and IP-10, have been recognized as potent chemo-attractant activators of neutrophils and T lymphocytes [8]. Both of them have participated in the pathogenesis of PDR.

To elucidate the potential value of angiogenesis-related cytokines and chemokines including VEGF, PEDF, IL-8 and IP-10, we collected serum samples of PDR patients before and after vitrectomy, and detected the cytokines concentrations by using ELISA. Herein, we aim to investigate the roles of angiogenesis-related cytokines in the pathophysiology of PDR progression.

SUBJECTS AND METHODS

Subjects

General information The study is a prospective, interventional, case control study. It was approved by the Institutional Ethics Committee of the Central Hospital of Wuhan. Informed consent was taken from every patient. Thirty DM patients with PDR were selected as experimental group. Thirty cases of age-matched healthy individuals were selected as control group. Patients were classified according to the presence or absence of DR and degree of severity using the final scale of the ETDRS Classification.

Ocular exclusion criteria were intraocular surgery, photocoagulation, uveitis and ocular trauma. For patients with photocoagulation done before, there was at least 3 months post laser time period for inclusion. Systemic exclusion criteria included ischemic cerebrovascular disorders, ischemic cardiovascular disorder, hepatic and renal dysfunction. Careful routine examination including visual acuity, intraocular pressure, slit lamp biomicroscopy and funduscopy were used to establish the diagnosis. Preoperative, intraoperative, and postoperative fundus findings were recorded for each subject. The severity of diabetic retinopathy was assessed by standardized color fundus photography and fluorescein angiography. During the vitrectomy, a standard three-port pars plana vitrectomy (PPV) was performed. Endolaser photocoagulation was performed, and when neovascular tissues were present, they were cut and removed.

Methods

Sample collection For controls, serum specimens were collected on admission. For PDR patients, serum specimens were collected before and 3 days after vitrectomy. All the samples were obtained in a sterile tube, placed immediately on ice, centrifuged at 2 000r/min for 5 minutes to separate the cell contents, and then rapidly frozen at -80℃ until processed. All serum specimens were collected in the morning hours after an overnight fast.

Table 1 Clinical characteristics of PDR patients

	Control group	PDR group
Cases/eyes	30/30	30/30
Male/female	16/14	18/12
Age(years)	40-75	38-73

Sample measurement Serum samples were thawed at room temperature and used for cytokine assays. For VEGF assays, serum samples were diluted 1:2 in a volume of 100μL. For PEDF assays, serum samples were diluted 1:10 in a volume of 100μL. The levels of VEGF, PEDF, IL-8 and IP-10 were measured by enzyme-linked immunosorbent assay (ELISA) using the Quantikine assay kit (R&D systems). The detection steps were referred to the test procedures of the kit. The optical density (ABS) of the final reaction plate was detected by amicroplate reader at 450nm wave length. The cytokine concentrations (pg/mL) of the samples were derived according to the standard calibration curve of various cytokines.

Statistics Analysis All the statistical analyses were done by SPSS version 10.0. Data were expressed as the mean±SD or median and range. Comparisons of groups were analyzed by independent samples t-test. Comparisons of data before and after vitrectomy were analyzed by dependent samples t-test. Statistical significance was defined as $P<0.05$.

RESULTS

Clinical Characteristics of PDR Patients Thirty patients with PDR were included in the study (30 eyes), of which male 18 eyes and female 12 eyes. The mean age of the diabetic patients was 52 years (38-73 years). The mean level of HbA1c was 8.0% (5.8%-9.9%), and the duration of diabetes mellitus was 12 years (Table 1).

Cytokine Profiles in PDR Patients All the serum concentration of various cytokines was shown in Figure 1. Serum concentration of VEGF was 168.45±59.23pg/mL in proliferative diabetic retinopathy, much greater than in controls (83.34±43.27pg/mL, $P<0.05$).The concentrations of PEDF, IL-8 and IP-10 in PDR group were also greater than that in controls (5.43±2.08 *vs* 3.12±1.51μg/mL, 105.54±36.22 *vs* 35.17±23.31pg/mL,132.12±35.78 *vs* 56.32±21.14pg/mL, $P<0.05$). The serum VEGF concentration decreased significantly in postoperative specimens than that in preoperative specimens ($P<0.05$), while the concentrations of PEDF, IL-8 and IP-10 did not exhibit significant difference.

DISSCUSION

In the current study, we measured several angiogenesis-related cytokines in the serum of PDR patients. Analysis of data *via* ELISA identified elevated serum concentrations of VEGF, PEDF, IL-8 and IP-10 in patients with PDR. It was also found that the VEGF level in the serum decreased

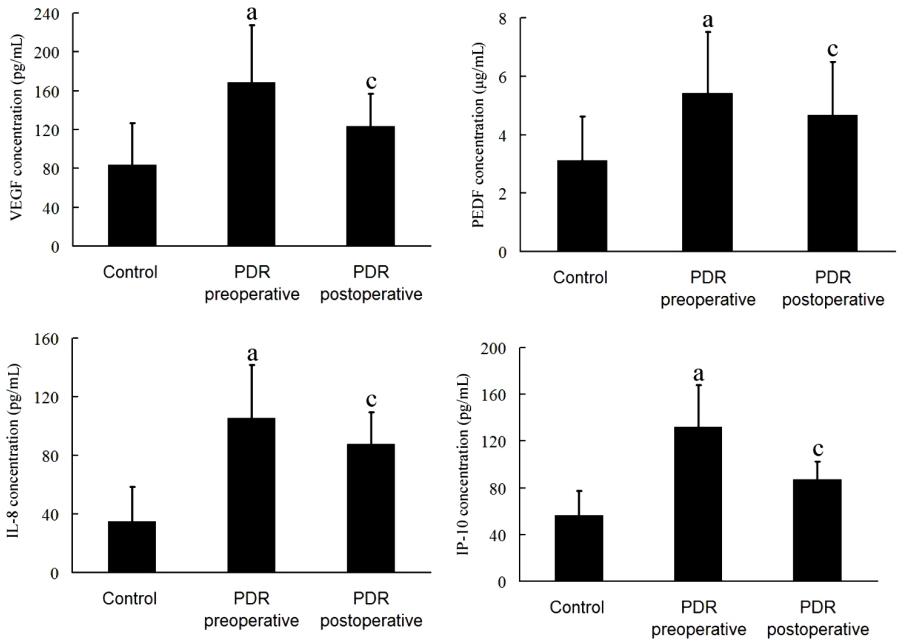

Figure 1 Cytokines concentrations in the serum of the control and PDR group [a]*P*<0.05 *vs*control; [c]*P*<0.05 *vs*PDR preoperative.

remarkably after vitrectomy.

Type 2 diabetes is considered to be a kind of chronic inflammatory disease. It can lead to severe microvascular complications, such as proliferative diabetic retinopathy and diabetic optic neuropathy. Studies showed that cytokine-mediated inflammatory response could induce insulin resistance and pancreatic B-cell apoptosis, which would contribute to the development of type 2 diabetes and the related complications.

VEGF is a multifunctional cytokine which increases microvascular permeability and directly stimulates endothelial cell growth and angiogenesis. In the pathogenesis of diabetic retinopathy, VEGF is the principal cytokine to promote angiogenesis, while PEDF acts as a main antagonistic component. VEGF can also stimulate the breakdown of the blood retina barrier, indirectly promote the progression of DR. In some researches regarding various cytokines, it has been demonstrated that hypoxia could induce VEGF expression *in vitro* and *in vivo*, and mediate ischemia-induced retinal neovascularization [9]. Moreover, it has been confirmed that the levels of VEGF in vitreous and serum of diabetic patients are much higher than those in health controls [10].

PEDF, which belongs to serine protease inhibitor gene superfamily, exhibits a variety of biological activity. In diabetic complications, PEDF exerts effects of neov-asculization inhibition, anti-inflammation, antioxidation, neurotrophy and neuroprotection [11,12]. PEDF was first purified from human retinal pigment epithelial cells, and was maintained high levels in retinal matrix. As the primary angiogenesis inhibitor, PEDF could restrain the growth and migration of retinal vascular endothelial cell, and contribute to the angiogenic homeostasis in ocular tissues. It can also inhibit retinal neovasculization induced by hypoxia, and promote retinal reparation against mechanical, light- and hypoxia-induced injuries. Also, PEDF could inhibit the proliferation and migration of endothelia cells induced by VEGF *in vitro*. It has been reported that VEGF could upregulate PEDF expression *via* VEGFR-1. In the present study, the results showed that serum VEGF and PEDF levels increased in PDR patients, which were in accordance with the previous studies. We infer that the elevation of serum PEDF levels in PDR correlated significantly with the changes of VEGF. The increase of PEDF might be due to the compensatory response to inhibit the increased VEGF.

It has been verified that vitrectomy relieves the retinal hypoxia in ischemic areas. Successful vitrectomy can inhibit the progression of retinal neovascularization in diabetic retinopathy[13]. The photocoagulation is associated with reduced neovascular activity. It may act on the outer retina including retinal pigment epithelium, and initiate some cascades to inhibit retinal neovascularization. Spranger *et al* [14,15] demonstrated that the VEGF concentrations in the vitreous of PDR patients decreased after retinal photocoagulation, while PEDF increased after the photocoagulation therapy. We speculate that vitrectomy can remove the vitreous and proliferative membranes and reduce the oxygen consumption of the retina. The retinal laser photocoagulation to the avascular area and new blood vessels can also destroy the photoreceptors; reduce the oxygen consumption of the outer

retina. Both of vitrectomy and photocoagulation reduce VEGF production in these areas and decreases new vessel formation. However, we could not detect significant concentration changes of PEDF after vitrectomy, probably owing to the insufficient effects of vitrectomy and retinal photocoagulation to the cytokine levels in serum. Considering the clues mentioned above, we supposed that the imbalance of pro- and anti-angiogenesis cytokines would contribute to the retinal neovasculization. At present, the anti-VEGF therapy towards diabetic retinopathy is provided with certain theoretical evidence. However, we inferred that the therapeutic effect of anti-VEGF only on diabetic retinopathy would be insufficient, because other cytokines except VEGF, especially some chemokines, also participate in the pathogenesis of PDR.

Chemokines belong to a superfamily of cytokines. They are micromolecular cell products which orchestrate the recruitment of leukocytes into the sites of inflammation. Recently, it was demonstrated that chemokines play pivotal roles in mediating angiogenesis and fibrosis [16]. IL-8 is a cytokine mainly produced by monocytes/macrophages, and is involved in the pathogenesis of various autoimmune diseases. Retinal vascular endothelial cells secrete IL-8 in the course of retinal vascularization [17]. Furthermore, retinal glial cells reactively secrete IL-8 in response to hypoxia [18]. It has been demonstrated that IL-8 increased in the hypoxic microenvironment of the diabetic retinopathy, and that IL-8 could mediate angiogenesis *via* both VEGF dependent and independent mechanisms [19,20]. In the vitreous of patients with PDR, increased levels of IL-8 have been found. Moreover, elevated vitreous level of IL-8 may be in association with large retinal vessel obliteration and related to ischemia [21]. In this study, the results showed that IL-8 increased in PDR patients. We supposed that elevated IL-8 level could be a marker of ischemic inflammatory reaction. Anti-IL-8 therapy would inhibit retinal neovasculization in PDR.

IP-10 is a kind of chemokine to promote the activation of T-helper cells and the migration of lymphocytes, which are signs of T cell response. Several studies reported that IP-10 is a potent inhibitor of angiogenesis and may have an inhibitory effect on fibrosis [22]. *In vitro* and *in vivo* studies have confirmed that VEGF could induce IP-10 expression in the pathogenesis of PDR [23]. The increased expression of IP-10 induced by VEGF would contribute to the main pro-inflammatory mechanism in the immune responses of PDR. A previous report documented elevated IP-10 levels in vitreous humor samples from patients with PDR [24]. Our study demonstrated increased serum levels of inflammatory cytokines IL-8 and IP-10 in PDR patients. However, the results in this study showed no significant difference of the serum IL-8 and IP-10 levels after vitreoretinal surgery. But it seemed undeniable that IL-8 and IP-10 participated in the inflammatory and immunological processes of PDR.

In conclusion, the serum levels of angiogenesis-related cytokines (VEGF, PEDF, IL-8 and IP-10) increased in PDR patients. Furthermore, the serum VEGF levels decreased greatly after vitrectomy. The angiogenesis-related cytokines may act as key regulators in the pathogenesis of PDR and provide potential tools for risk assessment in PDR patients. However, it still needs further studies to explore the effects and underlying relationships of various cytokines in diabetic retinopathy.

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