

Involvement of circulating endothelial progenitor cells and vasculogenic factors in the pathogenesis of diabetic retinopathy

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Abstract

Purpose Retinal neovascularization in diabetes has been thought to follow the release of local angiogenic factors in the retina. We hypothesize that neovascularization of diabetic retinopathy is a systemic vasculogenesis rather than a local angiogenesis. Thus, we evaluate the concentrations of circulating endothelial progenitor cells (EPCs) and stem cell modulation factors such as vascular endothelial growth factor (VEGF), erythropoietin (Epo), and substance p (SP) in the peripheral blood of diabetic retinopathy patients.

Methods We studied 15 normal controls and 45 type II diabetic patients (no DR group ($n = 15$), NPDR group ($n = 15$), and PDR group ($n = 15$)). We measured circulating CD34 + mononuclear cells (CD34 + MNCs), c-Kit + mononuclear cells (c-Kit + MNCs) by flow cytometry. VEGF, Epo, and SP in the peripheral blood were measured by ELISA.

Results The circulating CD34 + MNCs and c-Kit + MNCs increased in the NPDR and PDR groups compared with the control group ($P < 0.01$). Serum level of VEGF increased in the NPDR and PDR groups compared with the control group ($P < 0.05$). The level of Epo elevated exclusively in the no DR group compared with the other three groups ($P < 0.01$). Circulating SP level increased in the NPDR and PDR groups compared with the control group ($P < 0.05$). SP and CD34 + MNCs were shown to have increased correlation according to the diabetic retinopathy in the NPDR and PDR groups ($r = 0.440$, $P < 0.05$ and $r = 0.460$, $P < 0.05$, respectively).

Conclusions The present study is the first to demonstrate that CD34 + MNCs, c-Kit + MNCs and their modulator are elevated in diabetic retinopathy patients. Therefore, it is possible that circulating EPCs and serum Epo, VEGF, and SP may be involved in the progression of diabetic retinopathy.

Eye (2006) 20, 546–552. doi:10.1038/sj.eye.6701920; published online 20 May 2005

Keywords: endothelial progenitor cells; erythropoietin; diabetic retinopathy; substance p; vascular endothelial growth factor

Introduction

New vessel formation in diabetic retinopathy causes visual loss with vitreous haemorrhage, retinal detachment, and neovascular glaucoma.^{1,2} Retinal neovascularization has been thought to occur due to the release of local angiogenic factors by ischaemic and hypoxic retina.³ Increased local expression of vascular endothelial growth factor (VEGF) has drawn much attention relating to the pathogenesis of diabetic retinopathy. Evidences suggest that VEGF, a cytokine that acts as an endothelial cell mitogen,^{4,5} and induces increased microvascular permeability,⁶ is causally involved in the development of diabetic retinopathy.^{7,8}

Although intraocular levels of VEGF have been studied extensively, systemic vasculogenic factors have not been fully evaluated in previous studies. In particular, there appears to be a lack of strong association between systemic vasculogenic factors and diabetic retinopathy.

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Received: 11 October 2004
Accepted: 21 March 2005
Published online: 20 May 2005

Recent studies have demonstrated that circulating endothelial progenitor cells (EPCs) go to the sites of neovascularization and differentiate into endothelial cell *in situ* in a manner consistent with a process termed postnatal vasculogenesis.^{9–12} EPCs are characterized by CD34, Flk-1, and CD133 antigen-positive cells,^{13,14} and these cells are localized predominantly in the bone marrow.¹⁵ In the peripheral circulation of adults, more mature, differentiated EPCs are found that are characterized by a decreased expression of CD133, are positive for VEGFR-2, and still express CD34. EPCs circulate in adult peripheral blood and contribute to neovascularization, and their putative precursor, CD34 + monocuclear cells (CD34 + MNCs) increase in acute ischaemic events in human.¹² These EPCs are derived from adult bone marrow, and are known to be stimulated by several modulators such as VEGF, erythropoietin (Epo), substance P (SP), etc.^{9,16,17}

Sublethal hypoxia induces upregulation and nuclear translocation of hypoxia-inducible factor, whose target gene is Epo.^{18–21} Epo stimulates postnatal neovascularization at least in part by enhancing mobilization of EPCs from the bone marrow.^{22,23} Epo is also known to enhance mobilization of EPCs from bone marrow and maintain normal endothelial differentiation of retina on ischemic tissue to help the wound healing process.²²

Another modulating factor constitutes SP, which is a neuropeptide that could be released in the bone marrow in the ischaemic condition. SP interacts with specific receptors on resident cells in the bone marrow to modulate immune and haematopoietic functions.^{24–26} SP also has the same endothelial cell migratory effect at similar dosages as has VEGF, and induces vasoproliferation *in vivo*; that is, it has a vascular growth factor potential.²⁷

With this background, we hypothesize that neovascularization of diabetic retinopathy is a systemic vasculogenesis rather than a local angiogenesis, and EPCs and other stem cell modulators may be involved in the pathogenesis of diabetic retinopathy. We investigated whether circulating EPC precursors (CD34 + MNCs, c-Kit + MNCs) and other modulators (Epo, VEGF, SP) are changed with the progression of diabetic retinopathy, and tried to elucidate which vasculogenic factor is correlated with diabetic retinopathy.

Method

Study participants

We studied 45 type II diabetic patients and 15 age- and sex-matched normal controls. To prevent confusion with other systemic hypoxic conditions, patients were excluded if they had undergone prior ocular and cardiac surgery or had a history of ischaemic cardiovascular

disease (except hypertension), administration of statin or angiotensin-converting enzyme inhibitor, haematologic disorder, malignant disease, and human recombinant erythropoietin treatment that might influence EPC kinetics.^{15,28,29} Plasma fasting glucose, low-density lipoprotein cholesterol (LDL-C), and glycosylated haemoglobin (HbA1c) levels were assessed in all participants to address whether their blood glucose was controlled or not. All procedures were performed under the tenets of the Helsinki declaration and informed consent was obtained from all patients.

Grading procedure of diabetic retinopathy

Diabetic patients were classified into three groups according to the modified ETDRS retinopathy severity scale.³⁰ No DR group ($n = 15$) signifies no definite retinopathy in diabetic patients, NPDR group ($n = 15$) signifies nonproliferative diabetic retinopathy, and PDR group ($n = 15$) signifies proliferative diabetic retinopathy. Severity of diabetic retinopathy was confirmed by standardized fundus colour photography and fluorescein angiography.

Sample collection

Venous blood was collected from the antecubital vein and flow cytometric analysis was performed within 1 h of sample collection. For ELISA, samples were centrifuged at 3000 g for 5 min to obtain serum and then stored in a deep freezer at -70°C until they were assayed.

Measurement of circulating progenitor cells

Enumeration of CD34 + MNCs, stained with a reagent kit (ProCOUNT, BD) was measured by flow cytometer (FACSCalibur, BD).

Peripheral blood c-Kit + cells were identified by their distinct pattern of surface markers. C-Kit + cells were measured using c-Kit monoclonal antibody (BD Pharmingen) by the double-colour immunofluorescence technique.

Measurement of systemic vasculogenic factors

Serum levels of VEGF, Epo, and SP were measured with ELISA using human VEGF, Epo, and SP ELISA Kit (all from R&D Systems, Minneapolis, MN, USA). All procedures were performed following the instruction manuals.

Statistical analysis

Statistical analysis was performed with SAS statistical software (SAS Institute Inc., Cary, NC, USA). The differences among the groups were assessed by ANOVA followed by Duncan's multiple range test.³¹ Correlations between two variables were calculated by linear regression analysis. *P*-value <0.05 was considered to be significant.

Result

Demographic data of three diabetic groups

The data for age, sex ratio, and diabetes duration are shown as means in Table 1. There was no significant difference in age among the three groups of diabetic patients and the control group. The mean diabetes duration of 16.0 ± 6.0 years for the PDR patients was significantly longer than that for the NPDR and the no DR patients (11.3 ± 6.6 , 4.8 ± 2.3 , respectively; *P* = 0.02).

The mean fasting blood sugar (FBS) of the no DR patients (98.8 ± 7.0) was significantly lower than that of the NPDR and PDR groups (133.7 ± 21.3 and 143.2 ± 34.5 , respectively; *P* = 0.013). No significant differences were found in the prevalence of hypertension, atherosclerotic

cardiovascular disease, lipid profiles (esp. LDL-C), and HbA1c level.

Circulating progenitor cells in diabetic retinopathy

Table 2 shows levels of circulating CD34 + MNCs and c-Kit + MNCs in each group. The circulating CD34 + MNCs count gradually increased with diabetic retinopathy progression. Compared with the control group, the level of CD34 + MNCs significantly increased in the NPDR and PDR groups (*P* < 0.05 and < 0.001, respectively). Of the three DR groups, the level of CD34 + MNCs in the PDR group was significantly higher than that in the no DR group. C-Kit + MNCs, which reflects the total stem cell count including haematopoietic and endothelial lineage, significantly increased in the NPDR and PDR groups when compared with the control and no DR group (*P* < 0.0001). However, in the PDR group, c-Kit + MNCs decreased slightly compared to the NPDR group.

Systemic vasculogenic factors affecting mobilization of EPCs in diabetic retinopathy

Table 3 shows levels of serum VEGF, Epo, and SP in the control, no DR, NPDR, and PDR groups. Serum level of VEGF increased in the NPDR and PDR groups compared with the control group (*P* < 0.05). However, the level of VEGF among the three DR groups did not show a significant difference. Epo was exclusively elevated in the no DR group compared with the control group (*P* < 0.001). In the NPDR and PDR groups, Epo level showed no significant difference from that of the control group. There was a trend towards increasing serum SP level with the progression of diabetic retinopathy (*P* < 0.05). Especially, compared with the control group, the level of SP in the NPDR and PDR groups significantly increased.

Table 1 Data for age, sex, diabetic duration

	Control	No DR	NPDR	PDR
Number of patient	15	15	15	15
Age (years)	66.2 ± 16.1	68.4 ± 6.5	63.9 ± 6.2	66.9 ± 6.1
M/F	9/6	8/7	7/8	9/6
DM duration		4.8 ± 2.3	11.3 ± 6.6	16.0 ± 6.0

DR: diabetic retinopathy; NPDR: nonproliferative diabetic retinopathy; PDR: proliferative diabetic retinopathy; DM: diabetes mellitus. Values are mean \pm standard deviation.

Table 2 Result and statistical significance of circulating endothelial progenitor cells

	Control	No DR	NPDR	PDR
CD34 (cells/ μ l)	1.80 ± 0.54	1.90 ± 0.32	$2.52 \pm 0.72^*$	$3.13 \pm 1.06^{**}$
Duncan's multiple range test ^a	A	A B	B C	C
c-Kit (%)	0.88 ± 0.17	1.18 ± 0.62	$2.05 \pm 0.84^{**}$	$1.78 \pm 0.39^{**}$
Duncan's multiple range test	A	A	B	B

Values are mean \pm standard deviation.

P* < 0.05. *P* < 0.001.

^aResults with corresponding letters indicate that there is no statistical difference.

Table 3 Result and statistical significance of vasculogenic factors

	Control	No DR	NPDR	PDR
VEGF (pg/ml)	323±58	508±262	787±476*	616±301*
Duncan's multiple range test ^a	A	A B	B	B
Epo (mIU/ml)	7.63±1.75	13.44±1.30**	8.75±2.55	8.38±3.58
Duncan's multiple range test	A	B	A	A
SP (pg/ml)	69.8±35.4	72.3±29.2	122.7±72.1*	146.1±119.2*
Duncan's multiple range test	A	A B	B C	C

VEGF: vascular endothelial growth factor, Epo: erythropoietin, SP: substance P.

Values are mean±standard deviation. * $P<0.05$. ** $P<0.001$.^aResults with corresponding letters indicate that there is no statistical difference.

Correlation between vasculogenic factors and endothelial progenitor cells

To identify the relationship between factors in diabetic retinopathy progression, we performed a correlation test in three DR groups. There was no correlation between VEGF and EPCs. Epo was significantly correlated with CD34+MNCs only in the NPDR group ($r=0.6085$, $P<0.05$). SP was significantly correlated with CD34+MNCs in the NPDR and PDR groups ($r=0.440$, $P<0.05$ and $r=0.460$, $P<0.05$, respectively). VEGF level was inversely correlated with Epo level in the PDR group ($r=-0.61196$, $P<0.05$), but did not correlate with other parameters (data not shown).

Discussion

In this study, we found that circulating endothelial precursor cells (CD34+MNCs) were increased in the NPDR and PDR groups compared with normal control. Similar results reported that vasculogenesis-related progenitor cells were increased in coronary heart diseases.¹² Taken together with ours, these results suggest that retinal ischaemic signal may stimulate bone marrow by certain systemic factors that are diffused into systemic circulation, and CD34+MNCs were subsequently mobilized into peripheral blood and differentiated into endothelial cells on ischaemic foci. Even though these consequences of EPC elevation in diabetic patients are only speculative, our results have significance in the demonstration of EPC elevation in diabetic patients *in vivo*.

These results raise the question of whether elevated EPC levels in PDR patients play a beneficial role on ischaemic tissue or not. Previous reports demonstrated the therapeutic potential of EPC transplantation in

animal models of hindlimb and myocardial ischaemia.¹² However, EPC elevation seems to be less helpful or harmful to diabetic retinopathy. Tepper *et al*³² reported that diabetic EPCs exhibit alterations in functions important for blood vessel growth and may represent a mechanism by which diabetic EPCs are impaired in their ability to form collaterals. Neovascularization induced by altered EPCs may be structurally disorganized and insufficient for tissue oxygenation, thus elevated altered EPCs give rise to diabetic vascular complications. Based on this theory, we suggest that a supplement of normal healthy EPCs to the diabetic patients might prevent the progressive vascular changes.

Vascular endothelium as well as mesenchymal cells may be derived from bone marrow and contribute to organ regeneration and maintenance.⁹ C-Kit+MNCs reflect total stems, including mesenchymal stem cells. As the increase of c-Kit+MNCs was found to be less pronounced than that of CD34+MNCs in the PDR group, it might be speculated that an imbalance between endothelial and mesenchymal lineage cells cause excessive endothelial sprouting in the absence of mesenchymal architectural support. Thus, these vulnerable neovascularizations may lead to vitreous haemorrhage and release of angiogenic cytokines into the vitreous cavity.

Many of the diabetic patients who have impaired blood vessel growth after ischaemic events also have increased retinal neovascularization in diabetic retinopathy. This so-called 'diabetic paradox' is at present poorly understood.^{33,34} We could presume a plausible solution for diabetic paradox via EPC dysfunction and imbalance of circulating stem cells.

In this study, VEGF significantly increased in the peripheral blood especially in the NPDR and PDR groups. However, VEGF level did not correlate with the

severity of diabetic retinopathy. Similar results have reported that there was no difference in plasma immunoreactive VEGF level in type II diabetic patients,³⁵ and they found no association of serum VEGF level with retinopathy status in a larger number of diabetic patients.³⁶ Moreover, serum VEGF level did not correlate with CD34 + MNCs and c-Kit + MNCs in this study, which suggests that other unrecognized factors could be present, affecting the initiation of neovascularization and modulation of EPCs in diabetic retinopathy.

Epo is known to enhance mobilization of EPCs from bone marrow and to maintain normal endothelial differentiation of the retina on ischaemic tissue.²² Epo in diabetic retinopathy was first described by Berman and Friedman.³⁷ They reported three cases in which resorption of foveal hard exudates was observed after initiation of recombinant Epo treatment in anaemic patients. Friedman *et al*³⁸ reported that an increased haematocrit, induced by Epo, might slow the progression of diabetic nephropathy and improve visual acuity because of resolution of macular oedema in diabetic retinopathy.

In this study, Epo was significantly elevated in the early diabetic group with normal fundus findings, and decreased in the NPDR and PDR groups. These results suggest that Epo may be upregulated in the early stage of ischaemic disorders to maintain homeostasis against progressive diabetic retinal vascular changes. Epo may act as an endogenous neuroprotective factor against ischaemic retinal disorder. However, vascular change may begin as Epo level decreases according to the renal dysfunction.

Epo level was positively correlated with CD34 + MNCs count only in the NPDR group. This result may represent that Epo stimulates mobilization of EPCs from bone marrow in early diabetic retinopathy. Whether Epo administration to diabetic patients prevents progression of retinopathy, or whether Epo level may be used as a biologic indicator for early detection of diabetic retinopathy needs to be elucidated.

Another unrecognized factor may be SP, which has endothelial cell migratory effect,²⁷ and induces vasoproliferation *in vivo*; that is, it has a vascular growth factor potential.³⁹ Troger *et al*⁴⁰ reported that SP acts as a proangiogenic signal; thus, if SP is reduced, neovascularization does not occur even though VEGF and its receptors are upregulated. This fact can account for our result that VEGF level in the PDR group is less elevated than in the NPDR group. Even though VEGF level is not increased, elevated SP (proangiogenic signal) may induce neovascularization. SP shows positive correlation with CD34 + MNCs in the NPDR and PDR groups. We predict that SP may be responsible for transducing ischaemic signal, which is stimulated by

ischaemic damage of the microangiopathic retina, and may upregulate other vasculogenic factors and EPCs to promote neovascularization in ischaemic tissue. However, EPCs in diabetes are altered in function and make vulnerable vasculatures, upregulation of vasculogenic factors do not abate ischaemic signal, and SP may sustain high levels especially in proliferative retinopathy patients.

Many studies have been performed to identify the association between intraocular level and peripheral blood level of vasculogenic factors.^{41–44} Most of the results on VEGF showed a higher level in the vitreous than in the peripheral blood. However, there were few associations between intraocular level and peripheral blood level. In the case of Epo and SP, some reported that Epo and SP was elevated in the vitreous in inflammatory condition, but no studies were performed to confirm the association with systemic level. In the process of our study, we hypothesized that these vasculogenic factors increased in the eye diffuse into the systemic circulation and reach the target organ, bone marrow and stimulate EPC mobilization into systemic circulation.

There have been no studies that attempted to explain a progression of diabetic retinopathy incorporating vasculogenesis rather than just local angiogenesis. In this study, increased CD34 + MNC and c-Kit + MNC levels suggest that circulating vasculogenic factors may play a role in the progression of diabetic retinopathy. The present study is the first to demonstrate that systemic vasculogenic factors are elevated in diabetic retinopathy patients. Therefore, it is possible that systemic circulating EPCs and serum Epo, VEGF, and SP may be involved in progression of diabetic retinopathy. However, the precise functional roles of EPCs and modulating factors in diabetic retinopathy should be determined further in an *in vivo* study. Identification of such vasculogenic triggering factor and detection of an early biologic marker for diabetic retinopathy may help us to prevent progression of neovascularization and lead to the development of other therapeutic approaches, in order to improve the outcome of patients with diabetic retinopathy.

Acknowledgements

We thank J Kim for critically reading the manuscript and A Park for technical support. This study was supported in part by research funds from the Research Institute for Biochemical and Pharmaceutical Science, by Chung-Ang University and in part by research funds from the Ministry of Commerce, Industry and Energy, Republic of Korea.

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