Low content of the natural ocular anti-angiogenic agent pigment epithelium-derived factor (PEDF) in aqueous humor predicts progression of diabetic retinopathy

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Abstract

Aims/hypothesis. Retinopathy is the most common microvascular complication of diabetes. Our aim was to address the predictive value of pro-angiogenic and anti-angiogenic markers for progression of retinopathy.

Methods. Aqueous humor was collected at cataract surgery from 32 diabetic patients who had no or very mild retinopathy (ETDRS stage ≤ 20) and 33 normoglycaemic control subjects. Content of pro-angiogenic vascular endothelial growth factor and angiogenic inhibitor pigment epithelium-derived factor were determined. **Angiogenic** activity was quantified by measuring its effect on the migration of capillary endothelial cells. The predictive value of the initial level of these markers for progression of retinopathy was studied by following the probands for a maximum of 75 months.

Results. In the aqueous fluid content of vascular endothelial growth factor was increased in diabetic patients (mean values 492 versus 292 pg/ml; $p=0.0052$), and pigment epithelium-derived factor values were decreased (mean values 1740 versus 3680 ng/ml; $p=0.0058$) compared to control subjects. Of the diabetic patients ten progressed during follow-up (ETDRS

stage $>47B$). This subgroup showed lower pigment epithelium-derived factor content when compared to non-progressors and control subjects. Migratory activity in samples of patients from the control group and in diabetic patients without progression was generally inhibitory due to pigment epithelium-derived factor. Inhibition was blocked by neutralizing antibodies to pigment epithelium-derived factor. In diabetic patients initial angiogenic activity was higher in those who later developed retinopathy (vs. controls $p=0.00005$; vs. no progressors $p=0.0003$). Both pigment epithelium-derived factor and migratory response predicted progression.

Conclusion/Interpretation. Pigment epithelium-derived factor is an important negative regulator of angiogenic activity of aqueous humor. Its content in the aqueous humor of diabetic patients strongly predicts who among them will develop progression of retinopathy. [Diabetologia (2003) 46:394–400]

Keywords Angiogenesis, angiogenic inhibitors, aqueous humor, diabetic retinopathy, pigment epithelium-derived factor (PEDF), vascular endothelial growth factor (VEGF).

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Abbreviations: EBM, endothelial cell basal medium; ETDRS, early treatment of diabetic retinopathy study; PEDF, pigment epithelium-derived factor; VEGF, vascular endothelial growth factor.

Retinopathy is the most frequent chronic microvascular complication associated with diabetes mellitus. It represents a major threat to eyesight in Western countries, and is still the leading cause of blindness among people of working age [1]. Although intensive glycaemic control can delay the onset of diabetic retinopathy and ameliorate the severity of the disease, both in Type 1 and Type 2 diabetic patients [2, 3, 4], the incidence of diabetic retinopathy remains startlingly high [1, 5]. It eventually affects the majority of diabetics, progressing to legal blindness in about 5% of them [1, 5, 6].

Progressive diabetic retinopathy involves an angiogenic switch where normally quiescent vasculature begins to grow. Mild diabetic retinopathy does not pose an immediate threat to vision and can remain stable over many years. However, it constitutes a serious risk since it is an essential precursor to proliferative retinopathy, in which invasive vessels can compromise vision by growing into the ischaemic portions of the retina to cause detachment and/or entering the avascular vitreous to haemorrhage [1, 6, 7].

Despite intensive study of retinopathy in recent years, few have examined patients prior to the development of abnormal vessels or have followed such individuals prospectively. Much attention has been paid to the mechanisms of neovascularization in the later stages of retinopathy. Diabetic retinopathy, which is probably ischaemia driven, is accompanied by an increase in a number of angiogenic growth factors including hypoxia-induced vascular endothelial growth factor (VEGF) [7, 8, 9, 10, 11]. VEGF, which increases microvascular permeability and acts as an endothelial-cell selective mitogen, is considered a key factor in the disease [7, 9, 10]. Its content in the vitreous correlates with severity of the disease and antagonists of VEGF or its specific receptor ameliorate retinopathy in animal models [12].

However, the switch from quiescent to active vessels often involves not only an increase in inducers of neovascularization but also a decrease in the concentration of negative regulators of angiogenesis, thus resulting in the breaking of the delicate balance between angiogenic stimulators and inhibitors [13, 14]. One such natural inhibitor in the eye is pigment epithelium-derived factor [15, 16]. It is responsible for both the anti-angiogenic activity of human vitreous and for excluding vessels from the cornea [15, 16]. Regulated by oxygen concentrations, PEDF behaves in a manner opposite to stimulatory VEGF, falling in concentration when oxygen is limited and rising when it is in good supply [15, 17, 18, 19]. Recent data have provided evidence that PEDF is not only produced by the pigment epithelium but can also be found at high concentrations in most compartments of the eye, including the anterior chamber [20]. PEDF shows a dual function, firstly being antiangiogenic and secondly acting as a neutrophic factor promoting the survival of a variety of neuronal cells including photoreceptors [15, 16, 21, 22, 23].

To address the role of pro-angiogenic and anti-angiogenic factors in the early stages of diabetic retinopathy, we have determined aqueous humor content of the natural angiogenic inhibitor PEDF and of the angiogenesis stimulator VEGF. Also migratory activity in aqueous humor samples was obtained to address the functional role of pro-angiogenic and anti-angiogenic factors.

Subjects and Methods

Patients. Patients were recruited from the Department of Ophthalmology of the University Hospital in Ulm. Diabetic patients with cataract and non-diabetic patients with cataract only (controls) were recruited from January 1995 to December 1995, and followed until December 2001. Mean follow-up was 69 months (range: 64–75 months). Inclusion criteria were the presence of lense opacities that had to be treated by cataract extraction and lack of evidence for mild to severe non-proliferative retinopathy, i.e. ETDRS scale less than or equal to 20. Exclusion criteria included, uncontrolled hypertension (systolic values >170 mmHg, diastolic RR values >95 mmHg), ETDRS scale greater than 20 at baseline and uncontrolled diabetes (HbA_{1c} values >9%), to rule out probands that could show a so-called rebound phenomenon with an early worsening of diabetic retinopathy associated with lowering of blood glucose concentration [2, 3, 6, 24, 25, 26], macular oedema, including clinically significant macular oedema requiring grid laser photocoagulation. All patients signed written informed consent for their participation in the study. The study design was approved by the Ulm ethical committee.

Ophthalmologic follow-up. The patients were examined immediately after cataract extraction and aqueous humor collection. Examinations were repeated every 12 months during the follow-up period. The standard examination at every visit included measurement of visual acuity and measurement of intraocular pressure, examination of the anterior segment of the eye by slit-lamp biomicroscopy, gonioscopy to detect iris neovascularization, dilation of the pupil, direct and indirect ophthalmoscopy. Stereoscopic colour fundus photographs were taken every 12 months and were graded by observers masked to treatment and disease state. All members of the independent reading centre had been certified by the Fundus Photograph Reading Centre of the University of Wisconsin, Madison, USA. Grading of retinopathy was done according to the ETDRS scale. This scale divides diabetic retinopathy into over 20 levels ranging from absence of retinopathy (scale of 10) to severe vitreous haemorrhage in advanced proliferative retinopathy (score over 81). When indicated, laser photocoagulation was used according to standard recommendations [1, 6].

Collection of aqueous humor. At the onset of cataract surgery aqueous fluid from the anterior chamber of the eye was obtained. A maximum of 200 μ l (range: 50–200 μ l) of undiluted fluid was drawn under sterile conditions. The fluid was immediately frozen and stored at -80°C until assayed.

HbA_{1c} assay. HbA_{1c} was determined using the principles of ion exchange HPLC (BioRad, Munich, Germany; normal range: 4.3–6.1%).

VEGF assay. VEGF₁₆₅ was quantified using a commercially available sandwich enzyme immunoassay technique (R&D Systems, Wiesbaden-Nordenstadt, Germany).

Quantification of PEDF. Quantification of PEDF in clinical samples was carried out using immunoblots. To avoid loading errors samples were diluted 1 to 5 with saline and 5 μ l of the diluted sample was loaded resulting in 1 μ l of undiluted aqueous humor. Samples were run on at least two independent reducing SDS polyacrylamide gels along with serial dilutions of purified PEDF [15], transferred to PVDF membranes, blotted, probed with anti-PEDF affinity purified antibody [15] or probed with a monoclonal anti-PEDF antibody (Chemicon Int.,

Temecula, Calif., USA), and developed with a highly sensitive chemiluminescent kit (ECL kit, Amersham, Arlington Heights, Ill., USA). The blots were exposed to X-ray film, digital images obtained and quantified using ImageQuant densitometer (Molecular Dynamics, San Francisco, Calif., USA) and software, and PEDF concentration in the aqueous humor sample was calculated in relationship to the linear area of a standard curve generated using purified PEDF. Samples measured had been stored at -80°C for almost 4–5 years. Quality controls revealed no decay of PEDF values due to storage conditions used. Intra-assay and inter-assay precision less than 7%, respectively.

Boyden test: *In vitro* angiogenesis assay. Endothelial cell migration assays were carried out [15, 27] and samples were diluted 1 to 10 in serum-free endothelial cell basal medium (EBM) containing 0.1% bovine serum albumin, and added to the top side of gelatinized microporous membranes (8 μmol pore size, Nucleopore, Pleasanton, Calif., USA) in a modified Boyden chamber. Angiogenic activity was reflected by the ability of the samples to induce chemotaxis of neonatal human dermal microvascular endothelial cells (HMVECs from Clonetics Cell Systems, San Diego, Calif., USA), that had been plated at 10^6 cells per ml on the opposite side of the membranes. As endothelial cells lose their tissue-specific qualities upon culture and since PEDF can affect endothelial cells in the eye and control angiogenesis in the cornea, the system used reflects migratory response. Each sample was tested in quadruplicate. EBM with bovine serum albumin served as a negative control (BSA) and the same medium containing vascular endothelial cell growth factor (VEGF) at 200 pg/ml, as a positive control. Where indicated antibodies that had been affinity purified against a synthetic peptide and which do neutralize the anti-angiogenic activity of PEDF [15] were added at 20 $\mu\text{g}/\text{ml}$. The antibodies used had no direct effect on angiogenesis. Intra-assay and inter-assay variation was less than 7%, respectively.

Statistical analysis. Statistical calculations were done using the SAS package 6.12 (SAS Institute, Cary, N.C., USA). The Chi-square test was used for the analysis of categorical data. Student's *t* test was used for the comparison of VEGF-, PEDF-, and HbA_{1c} values. A logistic regression model was used to address and to verify the association between the markers determined at baseline as a single explanatory variable for progression of retinopathy. In the model other explanatory variables including age of the probands, HbA_{1c} values, diabetes duration and hypertension were also taken into account. A *p* value of less than 0.05 was considered statistically significant.

Results

Baseline characteristics. In 1995 1278 patients had cataract surgery, among them 358 were diabetic patients (28%). Out of 358 diabetic patients 94 met inclusion criteria, 32 diabetics and 33 control subjects were recruited for obtaining aqueous fluid and for subsequent ophthalmological follow up (Table 1). As expected, the HbA_{1c} values were higher in the group of diabetic patients. Sex, age, and cigarette smoking habits were comparable between the diabetic and the control cohort. The group of diabetic patients mostly comprised of Type 2 diabetic patients. All diabetic

Table 1. Characteristics of the patients at enrolment

Characteristics	Control subjects (n=33)	Diabetic patients (n=32)	<i>p</i> value
Sex			
Male	14	14	n.s.
Female	19	18	n.s.
Age (yr)			
Mean	71.7	72.2	n.s.
Range	54–85	55–90	
HbA_{1c} (%)			
Mean	5.56	7.19	0.001**
Range	5.0–7.3	5.1–9.0	
Type 1 Diabetics	n. a.	4	
Type 2 Diabetics	n. a.	28	
Insulin-treatment	n.a.	13	
OAD	n.a.	19	
Presence of Hypertension	14	21	0.071*
Cigarette smoking	7	7	n.s.

two non-diabetic probands showed a HbA_{1c} levels above the range of normal values (6.1%) with 6.6, and 7.3, respectively. However, glucose levels were found to be in the normal range. Hypertension was defined by a RR value equal to or above 140/90 mmHg and/or use of anti-hypertensive medication.

Wilcoxon rank-sum test for the comparison of continuous data.

* Chi-square test for categorical parameters.

n.s. = not significant

n.a. = not applicable

OAD = oral antidiabetic drug

probands had a good metabolic control with a mean HbA_{1c} of 7.19% (normal: $\leq 6.1\%$). In the control group (mean HbA_{1c} 5.56%) two subjects had a HbA_{1c} value above 6.1%, which is above the upper limit of normal. However, repeated blood glucose measurements showed normal blood glucose values in these subjects. Hypertension (as defined by a blood pressure of $\geq 140/90$ mmHg and/or use of anti-hypertensive drugs) was found in 42% (14/33) of the control subjects but it was higher in the diabetic cohort [66% (21/32); $p=0.071$]. At the end of the study period no remarkable change of HbA_{1c} values compared to baseline was found.

VEGF, PEDF, and HbA_{1c} values. Baseline VEGF content in the aqueous humor were increased in the cohort of diabetic patients ($p=0.0052$) with a mean of 493 pg/ml (range: 57–2000) as compared to the control group [mean value 286 pg/ml (range: 50–608)]. PEDF values tended to be lower in the diabetic cohort with a mean value of 1488 ng/ml (range: 10–5300) compared to 3890 ng/ml (range: 10–1970; $p=0.0058$) in the control group. The HbA_{1c} values showed no correlation to either VEGF or PEDF (Fig. 1, Fig. 2a).

Fig. 1. PEDF determination by use of Western blot analysis. In the upper part samples of aqueous humor are shown from control subjects, lanes 1–10; on the right side corresponding PEDF standards are given. In the lower part lanes 1, 3, 4, 6–10 correspond to samples from diabetic patients who showed progression, whereas in lanes 2 and 5 aqueous humor samples from non-progressors are shown

Progression of retinopathy – Prediction of retinal neovascularization. During the follow-up period, 10 of 32 (30%) diabetic patients showed progression of retinopathy and developed either pre-proliferative or proliferative lesions. No significant differences for age, diabetes duration, prevalence of hypertension, and HbA_{1c} values between the subgroups with and without progression could be found. All progressors showed an ETDRS stage greater than stage 47B, six showed an ETDRS stage greater than 53E. Of the diabetic patients, 22 showed no progression and remained below ETDRS stage 35. VEGF and PEDF values are shown in the subgroups with and without a progression of retinopathy (Fig. 2B). Mean value of VEGF was 447 pg/ml in patients with retinopathy and was not different compared to patients without progression. However, PEDF content was found to be lower in patients with progression when compared to

Fig. 2A, B. VEGF-, PEDF-values in aqueous humor. (A) baseline values of VEGF and PEDF in control subjects and diabetic patients. (B) baseline content of VEGF and PEDF values in control subjects and the subgroups of diabetic probands, i.e. patients with and without progression of diabetic retinopathy

Fig. 3. Angiogenic activity as determined in modified Boyden chamber assay. Overall angiogenic activity determined by induction of chemotaxis of human dermal microvascular endothelial cells. No difference was seen between control subjects and diabetic patients without progression to advanced stages of retinopathy. Angiogenic activity was increased in the aqueous humor of patients who progressed to retinopathy (progressors vs. controls: $p=0.00005$; progressors – non-progressors: $p=0.0003$).

both control subjects ($p=0.014$) and diabetic patients without progression ($p=0.013$).

In vitro angiogenesis assay. Endothelial cell migration assays were carried out to determine angiogenic activity as a marker of angiogenesis. Activity was reflected by the ability of the samples to induce chemotaxis of neonatal human dermal microvascular endothelial cells. VEGF, PEDF-values, and migratory activity are shown in progressors and non-progressors compared to control subjects (Fig. 3). Progressors showed an increased angiogenic activity compared to non-progressors as well as control subjects.

The mean number of cells migrating towards the samples from non-diabetic patients was $35.51 (\pm SD 8.01)$, and it was slightly higher ($38.9 \pm SD 6.33$) in diabetic patients without progression of retinopathy. However, the migratory response in the aqueous humor of the subgroup of diabetic patients that progressed to advanced stages of retinopathy in the following years was almost doubled, with endothelial cell migration rising to $64.63 (\pm SD 7.69)$. This difference was highly significant when compared to control subjects ($p=0.0005$), and when compared to the diabetic cohort without progression ($p=0.003$; Fig. 3).

PEDF controls angiogenic activity in aqueous humor. To determine, if PEDF controls the activity of angio-

Fig. 4. Endothelial cell migration assay: The aqueous humor of most non-diabetic patients was anti-angiogenic due to PEDF. Samples that showed no angiogenic activity when tested alone, were re-tested for the ability to block VEGF induced migration of capillary endothelial cells (clear bars). The same samples were tested with VEGF combined with antibodies that neutralized PEDF (shaded bars), to see if inhibition was relieved when PEDF was inactivated. 200 pg/ml of VEGF was added to stimulate migration maximally

genic stimuli in the aqueous humor of the eye, the samples from probands that had not been angiogenic when tested alone were further examined in the in vitro assay for the ability to block migration of endothelial cells towards exogenously added VEGF. Relevant controls showed that the blocking anti-PEDF antibodies used at 20 $\mu\text{g/ml}$ had no effect on basal or VEGF induced migration. Also purified PEDF at 500 ng/ml (10 nmol) effectively blocked migration towards VEGF and this inhibition was relieved as expected by anti-PEDF antibodies. The amount of VEGF added (200 pg/ml) is known to be able to stimulate vascular endothelial cells in vitro maximally. The majority of these samples turned out to be anti-angiogenic in that they were capable of inhibiting endothelial cell migration up a gradient of VEGF (Fig. 4). In each cohort were some samples that were not inhibitory and the proportion of these was slightly greater in the diabetic non-inducing samples, but this difference was not significant.

This inhibitory effect was due to PEDF as it was lost upon addition of an affinity-purified antibody known to neutralize anti-angiogenic activity of PEDF (Fig. 4). This reversal of inhibition caused by removal of the angiogenic inhibitor PEDF was significant compared to baseline activity both in the non-diabetic cohort as well as in the diabetic cohort ($p=0.001$; $p=0.01$, respectively).

Predictive value of parameters tested. A logistic regression analysis was carried out to verify the association between angiogenic stimulators and angiogenic

inhibitors as predictive markers for the progression of retinopathy. All variables used were defined at baseline; age, HbA_{1c}, with or without hypertension, as well as diabetes duration and were not associated with progression. The only variables that could be identified as predictive were the content of PEDF ($p=0.03$), as well as in vitro angiogenic activity ($p=0.024$) and the ratio of VEGF compared with PEDF ($p=0.043$).

Discussion

This study has addressed the roles of the angiogenic stimulator VEGF and the strong negative regulatory factor PEDF in the early stages of diabetic retinopathy in man. The analysis of samples from diabetic patients with no signs of advanced stages of diabetic retinopathy at baseline enabled us to determine pre-disease values of these mediators. By following the probands for almost 75 months it was found that PEDF-content was of predictive value for a significant progression of retinopathy. By use of the Boyden test we showed that angiogenesis was directly regulated by the PEDF content of the sample. A low content of the endogenous inhibitor PEDF in the aqueous humor predicted progression, whereas VEGF content was of no predictive value. The lack of association with classical risk factors for progression (i.e. HbA_{1c}, hypertension, and diabetes duration) observed in our study might have been due to the selection of probands and due to the number of subjects recruited.

As expected from the studies of others analyzing vitreous fluids obtained during active proliferative disease [9, 10], we observed that aqueous humor in the diabetic state, even prior to the onset of retinopathy according to standard clinical criteria, is characterized by an increase of the physiologically relevant angiogenic stimulator VEGF. Yet VEGF content in aqueous humor could not predict progression to pathological neovascularization, nor were VEGF values related to the ability to induce migration. These data suggest that a rise in stimulatory factors in the early stages of diabetic retinopathy by itself might not be sufficient to induce the angiogenic switch thought to lead to progression of diabetic retinopathy [13, 14]. It seems that a reciprocal fall in inhibitory PEDF is also needed. It seems likely that PEDF acts as a molecular brake on VEGF and other angiogenic stimuli and it is only when this brake fails that pathological retinopathy can develop [15, 16, 20].

Our in vitro data show that, like the vitreous, the aqueous humor from normal individuals is typically anti-angiogenic due to PEDF [15, 20]. This inhibitory activity is maintained in the majority of diabetic patients prior to onset of retinopathy but is lost in patients that have progressed significantly [20, 28, 29, 30, 31]. Recently an unbalanced expression of VEGF and PEDF in ischaemia-induced retinal neovascular-

izations was shown [20]. Most importantly, this showed that systemic treatment with purified PEDF protein can completely ameliorate ischaemic retinopathy in a mouse model of retinopathy of prematurity [33]. Such efficacy suggests that PEDF or molecules mimicking its effect could provide a new way of treatment for a wide variety of diseases of the eye that involve pathological angiogenesis, vascular remodelling and vascular leakiness. The regulatory function of PEDF was recently found to be due to the specific dependence of remodelling vessels on Fas/Fas ligand-mediated apoptosis [34].

Here we have provided clinical evidence that the concentration of the natural anti-angiogenic protein PEDF was of predictive value in the context of progression of diabetic retinopathy. A low value of PEDF predicted the development of the clinical signs of either pre-proliferative or proliferative retinopathy. PEDF, mimics of PEDF or other regulatory molecules of neo-angiogenesis could also be of considerable interest as novel treatment modalities in order to inhibit ocular neovascularization and delay progression to retinopathy [20, 33, 36, 37, 38, 39].

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