Systemic Upregulation of PDGF-B in Patients With Neovascular AMD

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Purpose. To determine the plasma levels of platelet-derived growth factor-B (PDGF-B), VEGF, and TNF-α in patients with neovascular AMD and in patients with diabetic macular edema (DME).

Methods. Thirty patients with neovascular AMD, 30 patients with DME, and 12 healthy controls were included in this prospective study. The concentrations of PDGF-B, VEGF, and TNF-α were measured by ELISA.

Results. The PDGF-B concentration in the plasma of controls was (median [25th–75th percentile]) 263.5 (162.0–513.3) pg/mL and in patients with DME 219.0 (122.8–604.8) pg/mL. In patients with neovascular AMD, PDGF-B levels were significantly higher with a median plasma concentration of 783.5 (289.3–1183.5) pg/mL ($P = 0.003$). The VEGF concentrations in patients with DME 33.0 (21.8–73.0) pg/mL and in patients with neovascular AMD 55.0 (37.0–116.3) pg/mL showed no significant differences ($P = 0.159$). A positive correlation of PDGF-B and VEGF plasma levels was found in patients with neovascular AMD and in patients with DME ($r = 0.683$, $P < 0.001$, and $r = 0.612$, $P < 0.001$, respectively). No significant differences of systemic TNF-α levels could be found between the three study groups.

Conclusions. Patients with neovascular AMD have significantly higher plasma PDGF-B levels compared with patients with DME and healthy controls. Our study data indicate that PDGF-B may be involved in the pathogenesis of neovascular AMD. (https://eudract.ema.europa.eu number, EudraCT 2010-024654-11)

Keywords: PDGF-B, TNF-α, HIF-1, neovascular age-related macular degeneration, diabetic retinopathy, plasma levels

Age-related macular degeneration and diabetic retinopathy (DR) constitute the most common causes of severe vision loss in developed countries. In both diseases, induction of the hypoxia-inducible factor-1 (HIF-1) cascade, which causes upregulation of several vasoactive gene products including, VEGF and platelet-derived growth factor-B (PDGF-B), is a key feature.[1]

In the last decade, great progress has been made in the treatment of these two pathophysiologically different disease entities by the development of VEGF antagonization. The implication of the HIF-1 signaling pathway focused attention on other HIF-1 regulated genes. Currently, PDGF-B seems to be appearing as a promising new HIF-1–associated treatment target on the horizon. The PDGF family of ligands consists of disulfide-bonded dimers of polypeptide chains. Platelet-derived growth factor, especially the PDGF-B variant, is implicated in the development of retinal vasculopathies.[2,3]

In patients with neovascular AMD, preliminary study data showed that a PDGF antagonist with high affinity and specificity in the form of an aptamer (Fovista, formerly E10030; Ophthotech Corp., Princeton, NJ) resulted in a significantly better visual improvement in combination with ranibizumab (Lucentis; Novartis Pharma AG, Basel, Switzerland and Genentech Inc., South San Francisco, CA) than anti-VEGF monotherapy alone (Boyer DS, et al. *IOVS* 2009;50:ARVO E-Abstract 1260). The PDGF-B/PDGF receptor system is important for the maintenance of vascular integrity and stability. Platelet-derived growth factor is a ubiquitous growth factor that stimulates pericyte recruitment and subsequent association of pericytes with the endothelial cells.[4,5] Pericytes are required for normal vascular stability and function.[6,7] During the development of neovascularization the coating of endothelial cells by pericytes is initiated by endothelial cell expression of paracrine PDGF-B, which forms the homodimer PDGF-BB.[8] The process of coating with pericytes causes a stabilization of the newly formed vessel and is characterized by a decreased sensitivity to anti–VEGF-A therapy. The stripping of pericytes from the newly formed vessel wall following the inhibition of PDGF-B signaling can render the endothelium more susceptible to VEGF blockade and thus may lead to regression of the neovascularization and improved visual outcomes (Cousins SW, et al. *IOVS* 2009;50:ARVO E-Abstract 1261).

The pro-inflammatory cytokine TNF-α might be another possible player involved in the development of AMD and DR It has been shown that TNF-α directly activates the HIF-1 cascade and clinicopathological and genetic linkage analyses detected a strong association of inflammatory processes in the development of AMD.[9–11] In line with these observations overexpres-

sion of the pleiotropic TNF-α has been found in choroidal neovascular membranes (CNV) of eyes with AMD.[12] Regarding the pathogenesis of diabetes and its complications, endothelial dysfunction as observed in diabetic macular edema (DME) can be triggered by expression of TNF-α and the subsequent production of O_2^-. (super-oxide radical).[13] Tumor necrosis factor α has been shown to be consistently elevated in the vitreous of patients with advanced stages of DR.[14–16]

It has recently been shown, that intravitreal therapy with bevacizumab (Avastin; Genentech, Inc.) significantly affects plasma cytokine levels in patients with AMD and DME. This demonstrates the involvement of systemic cytokine concentrations.[17–19] The pharmacodynamic principle responsible for the systemic effects of intraocular VEGF inhibition with a monoclonal antibody might be due to the neonatal Fc receptor (FcRn). The FcRn modulates immunoglobulin G (IgG) transport and it has been established that the long systemic half-life of IgGs as compared with other proteins are due to their recycling and rescue from catabolic elimination by the FcRn via direct engagement of the Fc fragment. The Fc region equips immunoglobulins with the ability to be transported across cellular barriers and to persist in systemic circulation. The presence of FcRn may explain why bevacizumab and fusion proteins like aflibercept, both of which contain an Fc domain, are able to readily cross the endothelial and epithelial boundaries like the blood–retina barrier.[20,21]

The systemic blockage of TNF-α with inhibitors such as infliximab has been shown to be effective in the treatment of selected AMD cases and refractory DME in preliminary studies.[22,23] The purpose of the present study was to analyze the plasma levels of PDGF-B, VEGF, and TNF-α in patients with neovascular AMD and in patients with DR. To the best of our knowledge data have not been published of a prospective series determining differences in the systemic concentrations of these key mediators in the signaling pathway of the two most common retinal neurodegenerative disorders.

Materials and Methods

Subjects

This prospective study was elaborated according to the Declaration of Helsinki and was performed after approval from the institutional review committee of the Medical University Innsbruck (Innsbruck, Austria). Informed consent was obtained from all included patients. Thirty patients with neovascular AMD and 30 patients with type 2 diabetes and DME were included. Diabetic retinopathy was classified according to the international clinical DR disease severity scale.[24] Patients with proliferative DR were not included. All patients did not receive intravitreal injections for at least 3 months prior to inclusion. Eyes that had undergone vitrectomy were excluded. Subjects with chorioretinal abnormalities, inflammatory comorbidities, uncontrolled hypertension, and systemic vasoproliferative disorders were excluded. Patients with anti-inflammatory treatments like steroids were excluded.

Controls were established from 12 healthy participants without any history of systemic and other ocular pathologies; subjects with AMD, chorioretinal abnormalities, diabetes, hypertension, inflammatory comorbidities, and vasoproliferative disorders were excluded.

Collecting Blood Samples

Blood samples were drawn from all patients by venous puncture with minimal stasis in the morning. Glycosylated hemoglobin (HbA1c) was assessed in all patients. Results were reported as DCCT/NGSP-HbA1c (%) values.[25] For the ELISA assays, blood samples were collected in tubes containing EDTA. Centrifugation was done at 3000 rpm for 20 minutes within 1 hour after sampling. Plasma was stored at −20°C until the assay, which was done within 4 weeks after sampling.

ELISA Assays

Platelet-derived growth factor–BB, VEGF-A, and TNF-α plasma levels were determined by ELISA (Human PDGF-BB Quantikine ELISA Kit #DBB00, Quantikine VEGF ELISA Kit; R&D Systems Europe, Abingdon, UK; #DVE00, and Human TNF-α Quantikine ELISA Kit DTA00C; R&D Systems Europe) as described by the manufacturer. Briefly, 100 mL of assay diluents (RD1X for PDGF-BB, RD1W for VEGF, and RD1F for TNF- α) was added to each well of 96-well polystyrene microplates, then 100 mL of standard or samples (EDTA-plasma) was added to each well, mixed by gently tapping the plate frame for 1 minute, and incubated for 2 hours at room temperature. Afterwards, washing with wash buffer (400 mL) was performed three times, followed by addition of 200 mL of polyclonal antibody conjugate to each well, incubation for 2 hours at room temperature and washing again with wash buffer three times. Subsequently, 200 mL of substrate solution was added to each well, incubated for 25 minutes at room temperature and finally, 25 mL of stop solution was added to each well. The concentration was determined by an ELISA reader at 450 nm. For TNF-α measurements the minimum detectable dose (MDD) defined by the manufacturer was 1.6 pg/mL. The percentage of TNF-α results falling below the limit of sensitivity is stated for each study subgroup, and calculations were based on observations above the detection limits. All ELISA readings of PDGF-BB and VEGF measurements were within the respective detection limits.

Statistical Analyses

All statistical analyses were performed using SPSS 19.0 (International Business Machines Corporation, Armonk, NY) statistical software packages. Continuous data are given as median and interquartile range (IQR) and qualitative data as percentages. The Kruskal-Wallis test and the χ^2 test were used for group comparisons. To test for normal distribution the Kolmogorov-Smirnov test was used. For the univariate analysis of covariance (ANCOVA) with age as covariate, the cytokine measurements were natural log transformed. After this normal log transformation the data yielded a normal distribution with acceptable skewness and kurtosis. Spearman's rank correlation analyses were used to determine correlation coefficients. For the statistical comparison of the correlations, z transformation according to Fisher was performed. P values less than 0.05 were considered to indicate statistical significance.

Results

The median PDGF-B concentration in the plasma of controls was 263.5 (162.0–513.3) (median [25th–75th percentile]) pg/mL and in patients with DME 219.0 (122.8–604.8) pg/mL. In patients with neovascular AMD PDGF-B levels were significantly higher with a median plasma concentration of 783.5 (289.3–1183.5) pg/mL ($P = 0.003$) (Fig. 1).

The median VEGF concentration in the plasma of controls was 35.0 (20.0–61.0) pg/mL. The median VEGF concentrations of patients with DME 33.0 (21.8–73.0) pg/mL and of patients with neovascular AMD 55.0 (37.0–116.3) pg/mL showed no significant differences ($P = 0.159$) (Fig. 2).

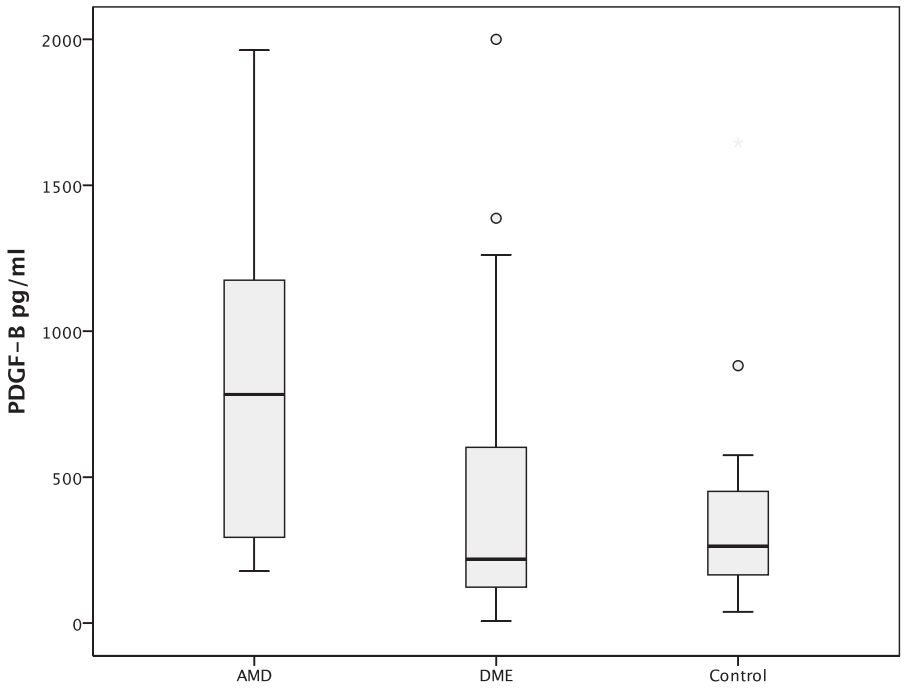

FIGURE 1. Box and whisker plots of PDGF-B measurements of the study cohorts.

A positive correlation of PDGF-B plasma levels and VEGF plasma levels was found in patients with neovascular AMD as well as in those with DME ($r = 0.683$, $P < 0.001$ and $r = 0.612$, $P < 0.001$, respectively). Correlation coefficient values did not reach statistical significance in the control group ($r = 0.575$, $P = 0.064$) (Figs. 3, 4). A z transformation showed that the correlations between the PDGF-B and VEGF plasma levels in the AMD and the DME groups did not differ significantly (zAMD = 0.8347, σAMD = 0.1925, confidence interval AMD [0.4574; 1.212]; zDME = 0.7121, σDME = 0.1925, confidence interval DME [0.3348; 1.0894]).

In the control group, the median plasma TNF-α concentration was 3.4 (2.4–5.1) pg/mL and results were below the detection limit in 58.3% of patients. The median plasma concentrations of TNF-α in the AMD and DME cohorts were 3.1 (2.6–4.1) pg/mL and 3.7 (2.6–4.7) pg/mL, respectively ($P =$

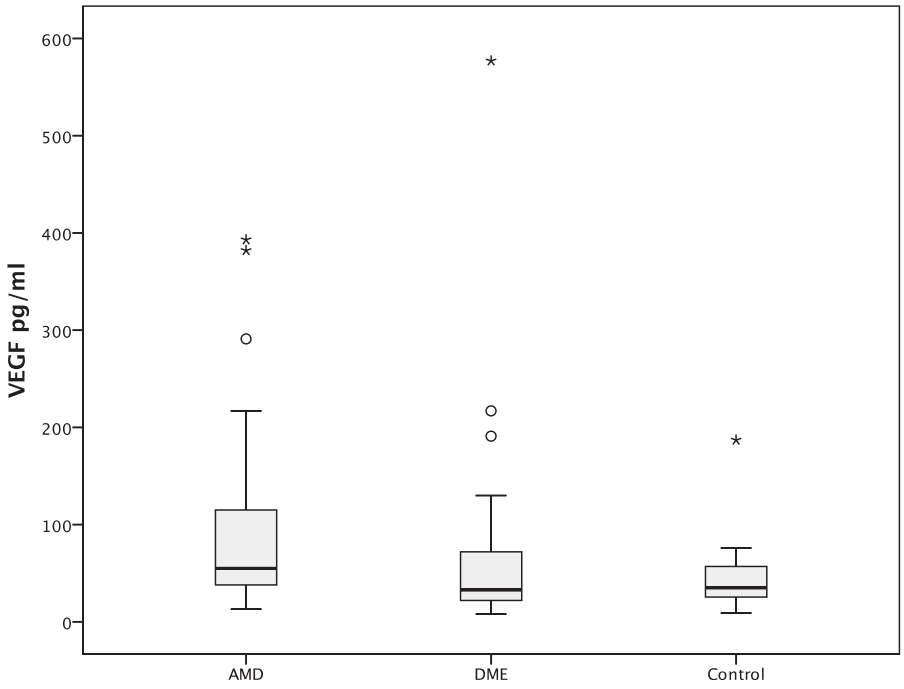

FIGURE 2. Box and whisker plots of VEGF measurements of the study cohorts.

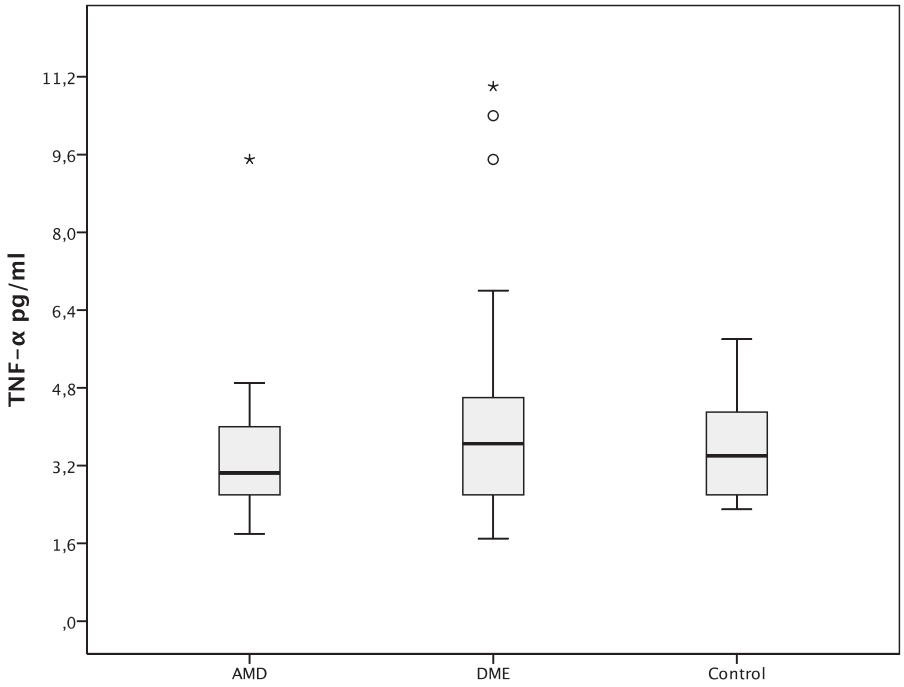

FIGURE 3. Box and whisker plots of TNF-α measurements of the study cohorts.

0.69) (Fig. 5). In patients with neovascular AMD, as well in patients with DME, TNF-α was below the minimum detectable dose in 26.7% of the patients. No differences were detectable between the control group and AMD/DME cohorts with respect to plasma TNF-α levels.

Details on demographics and plasma cytokine levels are summarized in the Table.

DISCUSSION

Given the potential importance of PDGF antagonization for the treatment of neovascular AMD it is surprising that currently no study data evaluating the plasma levels of PDGF-B in patients with AMD or DR have been published. The principle of antagonizing the cytokine VEGF had been developed nearly 10

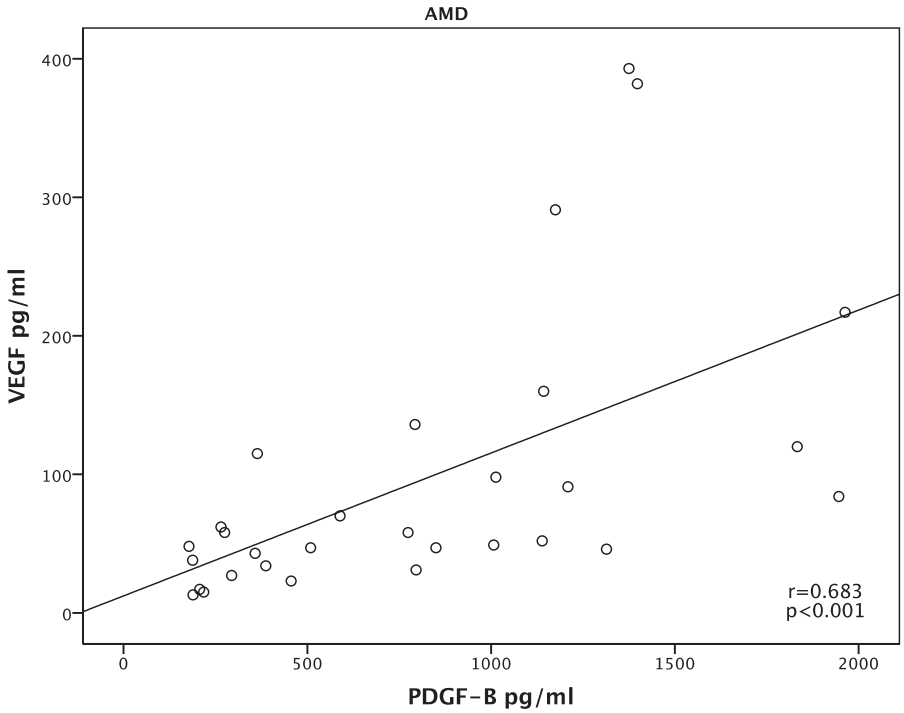

FIGURE 4. Scatter plot matrix of correlation between plasma PDGF-B and VEGF levels in patients with AMD ($r = 0.683$, $P < 0.001$; Spearman's rank correlation coefficient).

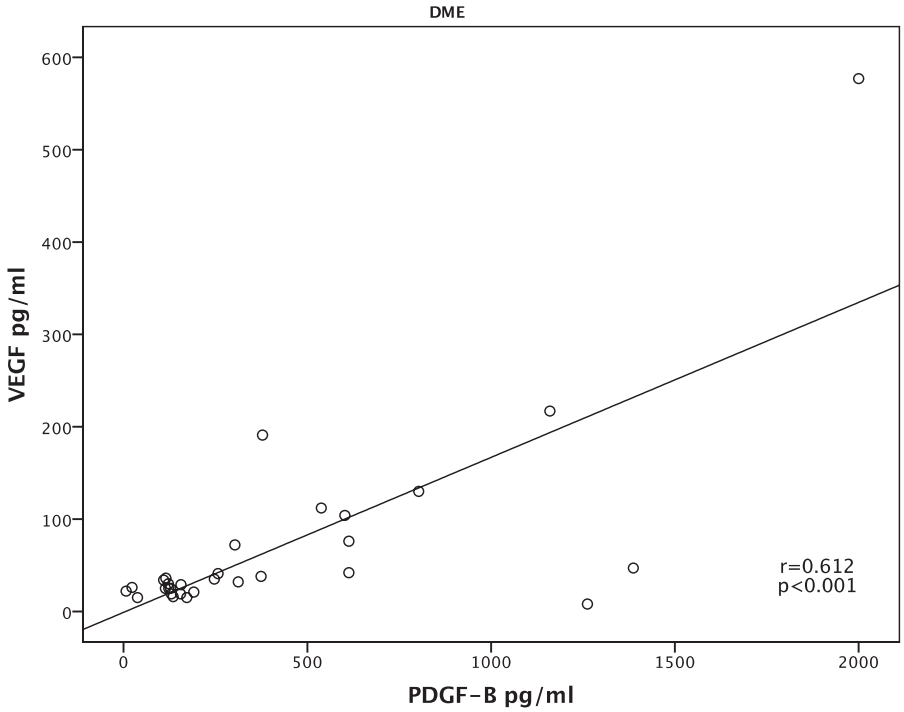

Figure 5. Scatter plot matrix of correlation between plasma PDGF-B and VEGF levels in patients with DME ($r = 0.612$, $P < 0.001$; Spearman's rank correlation coefficient).

years ago and is now firmly established as a mainstay of treatment for retinal vasculopathies. Vascular endothelial growth factor and PDGF are both members of a transcriptional cascade mediated by the transcription factor HIF-1, which accumulates under hypoxic conditions.[26] Currently, a new aptamer targeting the PDGF-B/receptor system is undergoing promising late stage clinical testing for the treatment of neovascular AMD (Boyer DS, et al. *IOVS* 2009;50:ARVO E-Abstract 1260). Our study shows significantly elevated systemic PDGF-B levels in patients with neovascular AMD compared with control as well as to patients with DME in nonproliferative DR.

Possible sources for the significantly increased PDGF-B levels in our study cohort of patients with neovascular AMD are either the active process of neovascularization in the choroid, or a systemic upregulation of PDGF-B expression. In vivo, PDGF-A and PDGF-B are produced at very low or undetectable levels in normal vessels, but PDGF and its receptors are detected in arteries and cultured vascular cells following injury.[27,28] Increased PDGF levels may represent a link between the pathophysiology of systemic vascular diseases and localized choroidal pathology as observed in AMD. Epidemiological studies have demonstrated several risk factors that are common to both cardiovascular disease and AMD and suggest that these disease entities may have similar pathophysiological mechanisms.[29,30] The possible common signaling pathways are subject to current research[31]; a positive association has been observed for atherosclerosis and AMD and the upregulation of plasma PDGF in patients with AMD might be consequential. Platelet-derived growth factor is strongly implicated in atherosclerosis by stimulating smooth muscle cell proliferation and migration resulting in an accumulation of neointimal smooth muscle cells and vascular stenosis and atherogenesis.[32] A possible pathophysiologic explanation is that endothelial cells exposed to fluid shear stress, which particularly occurs in the predilection areas for atherosclerosis, directly promotes PDGF transcription via induction of shear stress responsive elements.[33]

Table. Demographic and Plasma Cytokine Level Measurements of Patients

	Study Groups			
	AMD	**DME**	**Control**	***P* Value***
n (males *n*)	30 (10)	30 (21)	12 (5)	0.796
Age	79.0 (71.8–84.3)	66.0 (62.0–71.3)	48.0 (33.0–52.0)	<0.001
HbA1c, %	5.5 (5.2–5.9)	7.5 (6.7–8.5)	5.4 (5.1–5.6)	<0.001
PDGF-B, pg/mL	783.5 (289.3–1183.5)	219.0 (122.8–604.8)	263.5 (162.0–513.3)	0.003
VEGF, pg/mL	55.0 (37.0–116.3)	33.0 (21.8–73.0)	35.0 (20.0–61.0)	0.159
TNF-α, pg/mL	3.1 (2.6–4.1)	3.7 (2.6–4.7)	3.4 (2.5–5.1)	0.487
TNF-α detected, %†	73.3	73.3	41.7	0.078

Values are medians with 25th to 75th percentiles in parentheses. All measurement outliers are below the MDD.

* *P* values of less than 0.05 were considered to indicate statistical significance. Kruskal-Wallis tests, χ^2 tests, ANCOVA with age as a covariate for comparisons of cytokine concentrations.

† The MDD of TNF-α defined by the manufacturer was 1.6 pg/mL.

As atherosclerosis and other age-related vascular comorbidities can be expected in the study group with DME too, the active choroidal neovascularization (CNV) should be considered as another possible source of elevated PDGF-B levels in patients with AMD. It can be suggested that multiple pathways participate in the development of CNV. The initial stimuli leading to CNV have not yet been completely understood, but induction of the HIF-1 cascade, resulting in an upregulation of PDGF and VEGF-B expression, has a pivotal role in promoting the development of new vessels. The significantly positive correlation of VEGF and PDGF-B plasma levels in our study patients with AMD and DME might be attributed to their common transcriptional pathway.

The potential importance of upregulated PDGF levels in patients with AMD is affirmed by preliminary study data demonstrating the efficacy of cosuppression of PDGF-B, as well as VEGF; a combination of ranibizumab and an anti–PDGF-B aptamer has been shown to be more effective at inhibiting vessel sprouting than either individually. The combined therapeutic approach produced CNV lesion regression in 85% of eyes compared with only 20% in eyes with anti-VEGF monotherapy and, accordingly, resulted in significantly superior visual outcomes (Boyer DS, et al. *IOVS* 2009;50:ARVO E-Abstract 1260 and Cousins SW, et al. *IOVS* 2009;50:ARVO E-Abstract 1261). Further understanding for the superior efficacy of blocking VEGF, as well as PDGF can be achieved by considering the results of research in general medicine, more specifically oncology. A neovascular complex does not expand in a random fashion, but rather the angiogenic sprout expands led by tip cell filopodia. These are the only uncoated endothelial cells in the emerging neovascularization. The tip cells produce PDGF, which promotes attraction and coating with pericytes. The pericytes act as a protective armor for endothelial cells against anti-VEGF monotherapy by promoting endothelial cell survival through direct physical interaction as well as induction of maturation of the angiogenic sprout.[8,34] Vascular endothelial growth factor/PDGF interaction might be an important cause of resistance of the neovascular tissue to VEGF withdrawal.

Taking into account that the reasons for significantly increased plasma PDGF-B levels in patients with neovascular AMD remain unclear, further studies have to be performed, to examine possible side effects of anti–PDGF-B therapeutics for the treatment of neovascular AMD. Platelet-derived growth factor inhibition could interfere with physiologic pericyte function and result in a disturbance of the blood–retinal barrier, like observed in diabetic background retinopathy. It can be hypothesized that antagonization of PDGF in patients with DME might even result in a deterioration of the preexisting retinopathy and a further breakdown of the blood–retinal barrier.

Interestingly, the study patients with DME, contrary to those with AMD, did not show increased PDGF plasma levels compared to control. Though both CNV and DME respond well to VEGF antagonization, one must keep in mind that these two are different disease entities with contrary pathomechanisms, which may in part be reflected by the significantly different systemic PDGF levels observed in our series. In DR, pericyte loss is associated with increased vascular permeability and the formation of a range of retinal microvascular abnormalities, suggesting that PDGF inhibition may be appropriate for targeting CNV, but may not be suitable at all for the treatment of diabetic eye disease such as DME.[35–37]

A possible explanation for the significant difference of systemic PDGF-B levels in patients with AMD and DR may be a directly attributed to the effects of hyperglycemia in diabetic patients. As anticipated, the patients in the DR group had significantly increased HbA1c levels compared with subjects with AMD in our study. Glycosylated hemoglobin correlates well with average plasma glucose concentration and provides a moving average of blood glucose over the preceding 3 months.[25] Hyperglycemia affects the homeostatic interaction between pericytes and vascular endothelial cells. It was found that elevated glucose levels induce a cascade resulting in downregulation of PDGF production via increased expression of protein kinase C-δ (PKC-δ). Through this, hyperglycemia inhibits PDGF-B actions and induces pericyte apoptosis, which has long been established as a pathognomonic feature of diabetic vasculopathy.[38] Importantly, inactivation of the gene encoding PKC-δ has been shown to prevent the diabetes-induced loss of the blood–retinal barrier and subsequent vascular leak.[36]

Another factor that should be taken into consideration is that in the cohort of patients with DME none of the subjects were suffering from active proliferative DR. However for VEGF, as a key mediator of vascular proliferation, several studies have shown that plasma VEGF concentrations are not significantly correlated with the extent of diabetic microvascular complications.[39,40] Whether this lack of correlation is valid for systemic PDGF-B has not been studied in clinical trials so far and would be worth investigating.

Our results showed no differences in systemic TNF-α levels comparing the AMD and DR cohorts. Tumor necrosis factor α is a direct activator of the HIF-1 signaling cascade, which plays a fundamental role in the development of vasculopathy in AMD and DR.[9,11] Tumor necrosis factor α regulates a broad range of cellular activities including, proliferation, differentiation, and apoptosis.[9,41] In the eye, TNF-α is directly involved in the signaling cascades that induce breakdown of the blood–retina barrier, and recent studies have shown that TNF-α induces macular edema, retinal neovascularization, and proliferative vitreoretinopathy.[42–46] Increased expression of TNF-α causes apoptosis of various retinal neurons and leads to retinal neurodegenerative disorders.[47] As inflammation and hypoxia via induction of the HIF-1 cascade have been shown to play a role in both AMD and DR, we were not surprised to find no significant differences of systemic TNF-α levels in our study cohorts. In the control group the TNF-α levels were below the minimum detectable dose of 1.6 pg/mL in more than half of the subjects (58.3%). Values outside of detection limits generate biased statistical estimates, when less than half of measurements are within the boundaries. We therefore refrained from statistical analyzes of the relevant measurements and it can only be suspected, if TNF-α levels are lower in the healthy control group.

The limitations of the present study are that the cohort with healthy control subjects is not age-matched and the fact the concentrations of plasma factors may not be directly involved in the retinal proliferative or inflammatory process as such.

In summary, the present study shows significantly higher plasma PDGF-B levels in patients with neovascular AMD compared with patients with DME and healthy control subjects. Our study data indicate that PDGF-B may be involved in the pathogenesis of neovascular AMD.

Acknowledgments

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