Diabetic retinopathy is the leading cause of adult vision loss and blindness. The most important contributors to the development of diabetic retinopathy are hyperglycemia and hypoxemia that lead to increased vasopermeability, endothelial cell proliferation, and pathological neovascularization. In our previous studies, close relationship between proangiogenic activity of sera from type 2 diabetes mellitus patients (DM2) with background retinopathy, assessed in the in vivo serum-induced mouse cutaneous test (SIA), and VEGF and IL-18 serum concentration were observed. Moreover, it was clearly shown that IGF-1 might play an important role in the negative regulation of neoangiogenesis induced by DM2 patients' sera by diminishing the VEGF stimulatory effect. To confirm the observed phenomenon we evaluated the effect of DM2 patients' sera on the in vitro proliferative activity of human endothelial cells, which is critical for the sprouting and generation of new blood capillaries. Endothelial proliferative activity was significantly higher in the presence of sera from DM2 patients than from healthy controls (P<0.001), as estimated by the MTT test. Moreover, the examined sera from DM2 patients were characterized by increased IL-18 (P<0.05), diminished IGF-1 (P<0.02), and unchanged VEGF levels compared with those in controls. In conclusion, the present study showed a strong stimulatory effect of DM2 patients' sera on the proliferation of endothelial cells, which, along with the findings of our previous studies, proves that the described phenomenon is universal and valid for both animal and human endothelium.

**Key words:** diabetes type 2, endothelial proliferation, IGF-1, IL-18, retinopathy, VEGF
INTRODUCTION

Diabetic retinopathy is the leading cause of adult vision loss and blindness. The most important contributors to the development of diabetic retinopathy are hyperglycemia and hypoxemia that lead to increased vasopermeability, endothelial cell proliferation, and pathological neovascularization (1, 2). New blood vessels formation in the retina is also believed to be directly associated with increased expression of several proangiogenic cytokines, since ischemic retina secretes growth factors that are known stimulants of residual vessels proliferation (3, 4). Therefore, VEGF, bFGF, PDGF, TGF beta and IGF-1 have been implicated in the pathogenesis of retinopathy. Their elevated concentrations have been shown in the preretinal membranes of patients with proliferative diabetic retinopathy and in the patients' sera.

However, a causal bond between ischemia, growth factors and neovascularization has not been clearly demonstrated, despite a considerable research work. The majority of studies concerning this problem were performed in diabetes mellitus type 2 (DM2) patients with end-stage disease, demonstrating proliferative retinopathy, when malformations of retinal vessels became irreversible (5). Yet, in our opinion, investigation of an earlier stage of ocular complications, manifested as background retinopathy may prove most valuable in establishing the processes and factors contributing to retinopathy development. In our previous studies on DM2 patients with an early-stage ocular disease, close relationships between proangiogenic activity of their sera and VEGF as well as the IL-18 serum concentration were observed (6, 7). Moreover, it was clearly shown in the in vivo serum-induced mouse cutaneous test (SIA) that IGF-1 might play an important role in the negative regulation of neoangiogenesis induced by DM2 patients' sera by diminishing the VEGF stimulatory effect (8).

In order to confirm the observed phenomenon we decided to evaluate in vitro the effect of sera obtained from DM2 patients with background retinopathy on the proliferative activity of human endothelial cells, which represents the first, critical step in a sequence of events leading to the sprouting and generation of new blood capillaries. The concentration of several angiogenic cytokines - VEGF, IL-18, and IGF-1 also was assessed in the sera examined.

MATERIAL AND METHODS

The study was approved by a local Ethics Committee. Informed consent for blood drawing was obtained from each subject of the study according to the protocol approved by an institutional review board.

Patients' characteristics

The study was performed on sera collected from 12 healthy subjects (F/M - 6/6), aged 44-83 years (mean age - 66.1 ±3.8 years) and 12 DM2 patients (F/M - 6/6) with background retinopathy,
aged 47-85 years (mean age - 65.8 ±3.9 years). Retinopathy was diagnosed on the basis of fundoscopy performed by a skilled ophthalmologist and the worse eye was taken into consideration. The mean duration of DM2 was 17.2 ±3.4 years, HbA1c was 9.6 ±0.64% (normal range <6%). Patients and controls' sera after separation were aliquoted and stored at -78°C until use.

**Endothelial cell culture and proliferation**

The human umbilical vein endothelial cells (HUVEC) line was purchased from Cambrex (East Rutherford, NJ). Cells were grown to confluency in the EGM-2MV media in a humidified 5% CO2 atmosphere at 37°C.

Proliferative activity of HUVEC stimulated by sera of DM2 patients or controls was determined by MTT [3-(4, 5-dimethyl thiazol-2-yl) 2, 5-diphenyl-tetrazolium bromide] assay, as described by Mosmann (9) and Anuszewska (10). Briefly, HUVEC were diluted to 5 x 10⁵/ml in the EGM-2MV medium and cultured in this medium supplemented with 20% of the examined sera in 5% CO2 atmosphere at 37°C in 96-well plates (Costar, Acton, MA) for 72 h. Four cultures from each serum were established. Afterwards, cultures were treated with MTT (Sigma-Aldrich, St. Louis, MO) (5g/l in PBS) and incubated for another 4 h. Later, supernatants were removed, DMSO (dimethylsulphoxide) added into each well, and they were incubated for 5 min at room temperature. Absorbance was measured at 570 nm using a spectrophotometric reader Elx800 (Bio-Tek Instruments, Winooski, VT). The results from 4 cultures were pooled.

The stimulation index was expressed as a ratio:

\[
\frac{(A_{\text{contr}} - A_{\text{ex}})}{A_{\text{contr}}} \times 100%;
\]

where \(A_{\text{contr}}\) is absorbance in the culture with control sera and \(A_{\text{ex}}\) is absorbance in the culture with DM2 sera.

**Measurements of cytokine concentration**

Cytokine levels were determined in the examined sera using sandwich ELISA kits (R&D Systems, USA) for VEGF, IL-18, and IGF-1. Optical density was measured at 450 nm using a spectrophotometric reader Elx800 (Bio-Tek Instruments, Winooski, VT). Cytokine concentration was expressed as pg/ml.

Data were presented as mean (x) ±SE. Statistical analysis was performed using a \(t\)-test. A value of P<0.05 was considered to indicate statistical significance.

**RESULTS**

The proliferative activity of the endothelial cells cultured in the presence of sera from DM2 patients estimated in the MTT test was considerably higher in comparison with that of the cells grown with sera of healthy controls. The calculated mean proliferation stimulation index was 1.36 ±0.08 (P<0.001).

The mean concentration of VEGF in the DM2 sera was 449 ±51 pg/ml while in the control sera it was 375 ±108 pg/ml; the difference was not significant. The mean level of IL-18 was significantly higher in the DM2 than in control sera (453 ±43 pg/ml vs. 355 ±27 pg/ml; P<0.05), while the mean IGF-1 concentration was notably lower (85 ±10 pg/ml vs. 118 ±7 pg/ml, respectively; P<0.02).
DISCUSSION

Proliferation of the endothelium at the microvascular level is considered the crucial step in a sequence of events that leads to the generation of new blood capillaries in retinopathy (11). Proliferating endothelium is in the lead of the formation of endothelial tubes that next develop into immature and fragile capillaries that in diabetic retina are highly permeable and leaky (12). Mature capillaries might eventually be formed through a further action of angiopoietin, an antipermeability and anti-inflammatory agent (13). However, in diabetic retinopathy angiogenesis is a compensatory process in response to insufficient tissue oxygenation (14). Homeostatic abnormalities lead to retinal nonperfusion and subsequent ischemia, which, in turn, causes neovascularization and disruption of the normal retinal vasculature with formation of immature, permeable microvessels (2-4).

Physiological control of endothelial cells’ proangiogenic activity (proliferation, migration, invasion) and of angiogenesis depends, in general, on a fragile balance between inhibitory and stimulatory factors in tissues. As mentioned above, several factors have been thought to play an important role in retinopathy development, such as VEGF, bFGF, PDGF, TGF beta, IGF-1, and others.

In the present study, sera of DM2 patients, containing increased concentrations of proangiogenic factors, proved to induce dynamic proliferation of endothelial cells, compared with those from healthy controls. We have previously shown that VEGF and IL-18 closely correlate with the DM2 sera proangiogenic activity, as assessed by the SIA test, although the serum VEGF level in DM2 patients is not statistically different from that in controls (6, 7). Similarly, in the present study, no statistical difference was observed in the VEGF concentration in peripheral blood between the DM2 and control groups, despite the definite difference in their influence on HUVEC proliferation. However, it should be mentioned that the elevated VEGF concentration is observed only intraocularly in retinopathy patients, while insulin is known for its ability to suppress the VEGF serum concentration (15, 16). Besides, despite its normal serum level, VEGF is considered a key vasoformative factor in the pathomechanism of retinopathy, due mostly to its potent mitogenic effect on endothelial cells, stimulation of collagenase production, hypoxia-dependent activation, and the induction of vascular permeability (15).

On the other hand, the IL-18 concentration in the examined DM2 sera was higher than that in healthy controls. A similar phenomenon and a close correlation with the angiogenesis stimulation index have been observed in our previous studies (7). Although literature reports concerning the IL-18 angiogenic activity are somewhat contradictory, we have shown that recombinant hIL-18 induces a strong neovascular reaction in the mouse skin and this effect is diminished by specific anti-hIL-18 antibodies (17, 18). We believe, as it also has been implicated by others, that IL-18 might play a dual role in angiogenesis regulation, depending
on the circumstances and cytokine milieu. It is expected that IL-18's antiangiogenic action might be connected with induction of interferon gamma and disturbed by the presence of TGFbeta1 (19).

The only potentially anti-angiogenic factor evaluated in this study was IGF-1. We observed the considerably lower levels of IGF-1 in DM2 patient sera than in those from healthy controls. This observation is in accordance with our previous work showing not only a significant negative correlation between the IGF-1 concentration and DM2 sera angiogenic activity but also an inhibitory effect of IGF-1 on the VEGF-induced angiogenesis (unpublished observation). Yet, some literature data on the role of IGF-1 in the DM2 pathogenesis are contradictory and challenge our observations. Chantelau (20), for example, has strongly implicated the causative role of IGF-1 in the progression of retinopathy. However, his conclusion was based on the analysis of just four cases and clearly needs further studies. To complicate the picture further, it should be remembered that IGF-I is found in the blood in association with IGF-binding proteins (IGFBP) that may either inhibit or potentiate the bioavailability and consequently biological effects of IGF through several mechanisms (21). Moreover, it has been shown that IGFBP are able to exert IGF-1-independent effects that may be mediated by specific receptors, showing that the exact role of IGF-1 in the development of retinopathy remains enigmatic and definitely needs further investigation.

In conclusion, the present study examined the pro-angiogenic effects of sera from DM2 patients with background retinopathy. Similarly to findings of previous work on in vivo animal model of angiogenesis, we showed a strong stimulatory effect of such sera on the endothelial cell proliferation, proving that the described phenomenon is universal and valid for both animal and human endothelia. Moreover, DM2 patients' sera were characterized by increased IL-18, decreased IGF-1, and inappreciably changed levels of VEGF compared with those in control healthy subjects.

Acknowledgments: The research was supported by grant No. 3PO5B17223 from the State Committee for Scientific Research (KBN).

REFERENCES


Author's address: J. Chorostowska-Wynimko, Department of Molecular Diagnostics, National Institute of Tuberculosis and Lung Diseases, 26 Płocka St., 01-138 Warsaw, Poland.

E-mail: j.chorostowska@igichp.edu.pl