Serum *in vivo* angiogenic activity and some pro-angiogenic cytokine levels in diabetes mellitus type 2 (DM2) patients with or without background retinopathy

PIOTR SKOPIŃSKI [1,2], JERZY SZAFLIK [2], IWONA PARTYKA [2], JOANNA CHOROSTOWSKA-WYNIMKO [3], BARBARA DUDA-KRÓL [4], ANNA LIPIŃSKA [5], EWA SOMMER [6], EWA SKOPIŃSKA-RÓŻEWSKA [6]

[1]Department of Histology and Embryology, Center of Biostructure Research, Medical University of Warsaw, Poland; [2]Department of Ophthalmology, Second Faculty of Medicine, Medical University of Warsaw, Poland; [3]Laboratory of Molecular Diagnostics National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland; [4]Third Department of Internal Medicine, Second Faculty of Medicine, Medical University of Warsaw, Poland; [5]Department of Internal Medicine, Faculty of Dentistry, Warsaw, Poland; [6]Department of Pathology, Center of Biostructure Research, Medical University of Warsaw, Poland

Abstract

In our previous study we observed higher in vivo angiogenic activity and interleukin 18 level in sera of elderly patients with diabetes mellitus type 2 (DM2) and background retinopathy, in comparison to the controls.

The aim of the present study was to compare the serum IGF1, IL-18, and VEGF concentration as well as serum in vivo angiogenic activity in the diabetes mellitus type 2 (DM2) patients with (DM2R+) or without (DM2R-) background retinopathy and healthy controls. Moreover, the influence of human IGF1 on the angiogenic activity of sera obtained from healthy people and DM2 patients, and on the angiogenic activity of recombined human VEGF and IL-18 was estimated. Insulin-like growth factor 1 (IGF1) is described as partially responsible for diabetic retinopathy and renal vascular complications. However, its possible role in the negative regulation of angiogenesis in some selected physiological conditions has been mentioned as well.

Evaluation of angiogenic response was performed 3 days after intradermal injection of tested materials into mice skin (serum – or cytokine – induced angiogenesis, SIA, or CIA test). Estimation of VEGF, IL-18 and IGF 1 levels in tested sera were performed by ELISA.

Both DM2 groups presented higher angiogenic activity than that observed in the controls ($p<0.01$). IL-18 level was higher in DM2R+ group than in DM2R- group and than in the respective control ($p<0.05$). No differences in VEGF and IGF1 levels were seen. The addition of IGF1 to the sera of both DM2 patients and controls highly significantly diminished their angiogenic activity. Similarly, the new blood vessels formation stimulated by VEGF or IL-18 in the CIA test, was significantly lower in the presence of IGF1 ($p<0.01$).

Conclusions: a) in DM2 patients, serum IL-18 may serve as a marker of retinopathy appearance, b) in neovascular reaction evoked in vivo in mice skin after injection of human serum or human IL-18 and VEGF, human IGF1 behaved as angiogenesis inhibitor.

Key words: *DM2 patients, sera, angiogenesis, cytokines.*

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Correspondence: Piotr Skopiński, 2nd Department of Ophthalmology, Medical University of Warsaw, 13 Sierakowskiego Street, 03-709 Warsaw, Poland. Email: pskopin@wp.pl, ewaskop@hotmail.com

Introduction

Vascular endothelial growth factor (VEGF), potent angiogenic agent, has been implicated in the pathogenesis of retinal vasculogenesis and diabetic retinopathy [1]. Insulin-like growth factor 1 (IGF1) is also considered by many authors the angiogenic agent, partly responsible for diabetic retinopathy and renal vascular complications. The IGF1 peptide binds to the insulin/IGF family of receptors and activates their intrinsic tyrosine kinase domain activities, regulating number of biological responses [2]. Elevated intravitreal levels of IGF1 and VEGF correlating with neovascular activity were described, also interaction of IGF1 with IGF1 receptor promoting VEGF activity, was observed *in vitro* [3-5]. However, some authors described also alternative effects of IGF-1, suggesting its role in the negative regulation of angiogenesis in some selected conditions. Hui et al., Im et al. and Pattison et al. reported, that IGF1 blocks collagen release and down-regulates matrix metalloproteinases expression in chondrocytes, working alone, in combination with osteogenic protein-1, or together with transforming growth factor beta (TGF beta), partly by down-regulation of inflammatory cytokines [6-8]. It is noteworthy, as metaloproteinases are key factors in angiogenesis process. Also, Kajstura et al. observed that IGF1 overexpression inhibits the development of diabetic cardiomyopathy by interference with synthesis and secretion of angiotensin II [9], while in our previous study negative or slightly positive (depending on the type of diabetes) correlations between angiogenic activity of diabetic patients sera and their angiotensin converting enzyme (ACE) activity was observed [10].

We have previously demonstrated that human interleukin 18 (IL-18), cytokine belonging to the IL-1 family, as well as human VEGF and bFGF, revealed the ability to induce neovascular reaction in mice cutaneous angiogenesis test [11, 12]. Human IGF1, however, have not presented such property. In fact, neovascular reaction observed after intradermal injection of this cytokine was even less intense than reaction observed after injection of phosphate-buffered saline (PBS), what might suggest some inhibitory influence. Moreover, absorption of IGF1 from human serum with anti-IGF1 antibody before performing cutaneous test, have increased neovascular reaction (manuscript in preparation).

In our previous study [12] increased IL-18 content and higher angiogenic activity of sera collected from elderly DM2 patients with background retinopathy in comparison to the age-matched controls were observed. Absorption of sera with anti-IL-18 antibody resulted in substantial decrease of these sera angiogenic activity. Interestingly, Cho et al. recently reported that in rheumatoid arthritis (RA) patients IL-18 dose-dependently increased the production of VEGF [13].

The aim of the present study was to compare the serum IGF1, IL-18, and VEGF concentration, as well as serum *in vivo* angiogenic activity, in diabetes mellitus type 2 (DM2) patients with (DM2R+) or without (DM2R-) background retinopathy and healthy controls. Moreover, the influence of IGF1 on the angiogenic activity of sera obtained from healthy people and DM2 patients, and on the angiogenic activity of recombined human VEGF and IL-18 was estimated. Evaluation of angiogenic response was performed 3 days after intradermal injection of tested materials into mice skin according to the method described, serum – induced angiogenesis, SIA test [14, 15] and cytokine-induced angiogenesis, CIA test [11, 12].

It should be mentioned that, as we have demonstrated previously, human serum and some human cytokines (VEGF, bFGF, IL-18) injected intradermally into mouse skin induced strong neovascular response around the site of injection. Complement inactivation by heating of serum for 30 min at 56°C significantly diminished the number of newly-formed blood vessels. No influence of heating on the sera levels of VEGF, IGF1 and IL-18 and some decrease of bFGF concentration were observed [16].

Material and methods

The study group consisted of 30 healthy people, aged 40-83 years and 67 patients with type 2 diabetes mellitus (DM2) without proliferative retinopathy, 40-90 years old. The mean duration of diabetes mellitus was 12±1.6 years, HbA1c was 9.15±0.5% (normal range <6%). Background retinopathy was diagnosed in 37 of DM2 patients according to criteria of ETDRS (Early Treatment Diabetic Retinopathy Study). According to this diagnosis patients were included to DM2R+ or DM2R- groups. Informed consent for blood drawing was obtained from each subject according to an institutional review board-approved protocol and the study was approved by a Local Ethical Committee. Patients and controls sera after separation were aliquoted and stored at –78°C until further examination.

All sera were tested for *in vivo* angiogenic activity (mouse cutaneous test) and *in vitro* for VEGF, IGF1 and IL-18 content (ELISA).

Serum-(SIA) or cytokine-(CIA) – induced cutaneous angiogenesis assay

Cutaneous angiogenesis assay was performed according to Sidky and Auerbach method [17] with own modifications [12, 15, 18]. Studies have been performed in 2-month old, female inbred Balb/c mice. Mice have been of local laboratory breed, weighing ca 20 g each. The sera of healthy subjects or DM2 patients, or cytokines, were injected intradermally (0.05 ml per one injection, 3-6 injections per mouse) into regionally shaved, anaesthetized with chloral hydrate (POCH, Poland) groups of 3 or more mice. In order to facilitate the localization of injection sites later on, all injected samples were coloured with 0.1% of trypan blue. After 72 hours mice were killed with lethal dose of Morbital

(Biowet, Poland). All newly formed blood vessels were identified and counted in dissection microscope in 1/3 central area of microscopic field, at 6 x magnification. Identification was based on the fact that newly-formed blood vessels differ from background vasculature by their small size, tortuosity and divarications [17]. Mean number of newly-formed blood vessels was calculated from a dozen or so separate readings and designated as "angiogenic activity" of tested sample.

The advantage of mice cutaneous test in comparison to the mice corneal and other *in vivo* tests (matrigel, sponge) may be summarized as follow: short experiment (72 hours), more humanitarian than other tests, and more economical (4-6 readings from one mouse permits to obtain 12 to 18 readings from the group of 3 mice for further statistical evaluation).

Experiments were approved and supervised by the Local Ethics Committee.

Measurement of VEGF, IL-18 and IGF1 concentration

Cytokine levels were determined in examined sera using sandwich ELISA kits (R&D Systems, USA) for human VEGF, IL-18, and IGF1, according to the producer instructions. Optical density was measured at 450 nm using spectrophotometric reader Elx800 (Biotek Instruments, Inc., USA). Cytokines concentration was expressed as pg/ml.

Inhibition experiment

In separate experiment pooled sera samples obtained from 6 healthy people (control pool) and 6 DM2R+ patients (DM2 pool) were established. Both samples were heat--inactivated (30 min, 56°C) in order to remove complement. IGF1 (human rh IGF1, R&D) was mixed with respective serum samples (1:9 vol:vol), or 300 pg/ml of VEGF (Human VEGF, Biosource), or 300 pg/ml of IL-18 (human IL-18, R&D), to obtain the final concentration of 100 or 200 pg/ml of IGF1, and incubated for 60 min at 37°C and 5%CO_2 atmosphere before injecting into mice skin. As the control, sera samples or cytokines were mixed with phosphate buffered saline (PBS).

Statistical analysis was performed using Student-*t* and Mann-Whitney U tests.

Results

The results of SIA test and cytokine evaluation are presented on the table 1.

The mean angiogenic activity of sera from DM2 patients estimated by the SIA test was significantly higher in comparison to healthy controls (table 1), also when complement was removed by heating (table 2). No difference between DM2R+ and DM2R- groups was found so far as this activity was concerned.

Table 1. *In vivo* angiogenic activity (SIA test) and cytokines level (ELISA) in DM2 patients and control sera

		Mean cytokine concentration (pg/ml) ±SE		
source of sera	**mean number of newly-formed blood vessels ±SE**	**VEGF**	**IGF1**	**IL-18**
DM2 patients with background retinopathy	60.2±1.6 (360)**	316±48 (74)	86±11 (42)	418±27 (52)*
DM2 patients without retinopathy	68±1.7 (324)**	445±80 (52)	69±8 (48)	335±27 (32)
controls (without diabetes)	44.1±1.4 (324)	285±28 (60)	88±7 (36)	344±20 (52)

*( ) total number of readings in parentheses; * p<0.05; ** p<0.01.*

Table 2. Inhibitory effect of IGF1 on neovascular reaction induced in mouse skin by heat-inactivated human serum, IL-18 or VEGF

	Without IGF1		With IGF1 (100 pg/ml)		With IGF1 (200 pg/ml)	
	n	**mean number of blood vessels ±SE**	**n**	**mean number of blood vessels ±SE**	**n**	**mean number of blood vessels ±SE**
pooled sera from 6 healthy people	11	17.1±0.92	15	7.6±0.47**	12	6.7±0.55**
pooled sera from 6 DM2 {R+} patients	14	24.6±1	14	13.5±0.53**	11	7.6±0.47**
IL-18 300 pg/ml	14	11.3±0.69	14	8.6±0.45**	–	not tested
VEGF 300 pg/ml	14	13.8±0.51	16	9.7±0.62**	–	not tested
PBS (traumatic angiogenesis)	16	5.7±0.42	14	4.6±0.35	–	not tested

*n – number of readings; ** p<0.01.*

IL-18 level was higher in the DM2R+ group than in the DM2R- group and higher than in the respective controls. No significant differences of VEGF and IGF1 levels were seen between groups.

The effect of IGF1 on the sera and cytokines proangiogenic activity (table 2)

We confirmed the ability of human VEGF and human IL-18 to induce neovascular reaction in mice skin. However, we have not obtained again stimulatory effect of human IGF1 in this model of angiogenesis. Moreover, the addition of IGF1 to the pooled sera of both DM2 patients and controls significantly diminished their angiogenic activity ($p<0.01$). Similarly, the new blood vessels formation stimulated by VEGF or IL-18 in the CIA test, was significantly diminished in the presence of IGF1 ($p<0.01$).

Discussion

Neovascularization is one of the key sight-threatening processes than can occur during diabetic retinopathy. New blood vessels formation in the retina is strongly associated with the retinal ischemia, and several growth factors have been implicated in its pathogenesis. The ischemic retina is supposed to secrete the growth factors that stimulate residual vessels to proliferate. Histological studies have demonstrated their presence in the preretinal membranes of patients with proliferative diabetic retinopathy, while elevated concentrations of VEGF and IGF1 have been observed in sera and intravitreally [19-21]. According to the literature data, VEGF is the factor most closely correlating with new blood vessels formation, and its receptor blockade prevents retinal neovascularization [1]. VEGF concentration in sera of retinopathy patients evaluated in the present study was not different from the controls, while somewhat increased levels (on the border of statistical significance) of this cytokine were observed in sera collected from DM2R- patients. On the other hand, sera of DM2R+ patients contained highly significantly more IL-18 than sera collected from two other groups. This confirm our earlier study where we have found significantly more IL-18 in the sera of DM2R+ elderly patients than in the sera of aged controls [12]. However, in that earlier study we had not diabetic patients without retinopathy.

Mahmoud et al., as well as Nakamura et al. have previously reported elevated levels of IL-18 in the sera of DM2 patients with nephropathy suggesting that serum IL-18 is a predictor of disease progression in diabetic nephropathy [22, 23]. In our opinion it might have similar value in diabetic retinopathy. Recently, Suchanek et al. reported, that coronary artery disease (CAD) patients with DM2 had a higher concentration of IL-18 in their sera in comparison to the non-diabetic CAD group [24]. Elevated plasma IL-18 was also reported as a marker of insulin resistance in type 2 diabetic and non-diabetic humans [25].

In the present study we have found that admixture of IGF1 to the sera or to VEGF and IL-18 samples suppressed their angiogenic activity.

The literature data concerning the role of IGF-1 in the pathogenesis of DM are scarce and mostly challenge our observations. Chantelau strongly implicated IGF-1 causative role in the retinopathy progression, however his conclusion was based on the analysis of four cases only and clearly needs further evaluation [5]. Grant et al. described pro-angiogenic activity of Elvax pellets mixed with IGF1 and implanted into rabbits cornea [4]. However, in her study IGF1 activity was examined at very high dose (10 µg), substantially higher than amounts present in human serum and than concentrations used in our experiments (100-200 pg/ml, 5-10 pg in 0.05 ml).

It was demonstrated previously that blockade of the VEGFR signalling was sufficient to completely prevent retinal neovascularization [1], while inhibition of IGF1 by somatostatin analogs produced unsatisfactory results [21]. In enoxaparin-treated orthopaedic patients VEGF sera concentration after 14 doses of this drug have rose paralelly to their *in vivo* angiogenic activity [26] but no increase or decrease of IGF1 level was observed (manuscript in preparation). Those observations confirmed different functions of VEGF and IGF1 in angiogenesis, as suggested by our present results. Data presented by Im [7], Pattison [8] and Zhang [27] also supported dual effect of IGF1 on the activity of matrix metalloproteinases depending on cytokine milieu and ligand receptors bioavailability, that can shift the balance preferentially toward one or the other pathway.

In conclusion, present study demonstrates: a) higher angiogenic activity of DM2 patients sera in comparison to healthy controls b) higher IL-18 level in DM2R+ sera than in controls and DM2R- patients, c) no direct pro-angiogenic effect of human IGF1 in cutaneous test in mice, d) direct inhibitory effect of IGF1 on the *in vivo* angiogenic activity of human serum, VEGF and IL-18 in mouse cutaneous test.

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