Angiogenic activity and IL-12p40 concentration in healthy people and diabetes patients sera

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Abstract

Angiogenic eye disease is one of the most common causes of blindness. As IL-12 is known as quite strong anti-angiogenic factor, free IL-12 subunit: p40 is IL-12 antagonist and we aimed to determined its concentration and correlation with angiogenesis induced by sera of diabetic patients. We showed positive correlation between angiogenic activity of DM2 sera and their IL12p40 content. The mean number of newly formed blood vessels was significantly higher in DM2 patients as compared to control subjects. There were no differences between angiogenic activity in DM2 patients with and without retinopathy. Similarly we evaluated higher concentration of IL-12p40 in DM2 patients as compared to control group. No differences between patients with and without retinopathy were detected in IL-12p40 sera concentration. In case of DM1 patients angiogenic activity was lower as compared to healthy control patients, but IL-12p40 level was not different from the control.

Key words: *angiogenesis, diabetes mellitus, Il-12p40*

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Introduction

Angiogenic eye disease is one of the most common causes of blindness. Current therapeutical approaches are insufficiently effective and partially associated with serious adverse effects. The current understanding of angiogenesis has already led to the identification of a number of potential mechanism involved in pathophysiology of the disease still it is a quite unknown field.

Proliferative retinopathy in diabetes mellitus is one of angiogenesis dependent diseases.

Interleukin 12 (IL-12) is a multifunctional cytokine produced by macrophages and B-cells. It has the potential to induce interferon-gamma (IFN-γ) production, stimulate the growth of both T- and natural killer (NK) cells, promote Th1-type helper T-cell responses and inhibit neovascularisation in solid tumors [1, 2]. Moreover, IL-12 has also been found to inhibit cytokine-induced neovascularisation. [3]. In that study, measurement of maximal vessel length and corneal circumference was used to assess both the induction of angiogenesis, by basic fibroblast growth factor, and its inhibition by IL-12 or IFN-γ. The authors postulated that this inhibitory effect was mediated via IL-12 induction of IFN-γ, which, in turn, appeared to play a critical role as a mediator of the anti-angiogenic effects of IL-12.

As IL-12 is known as quite strong anti-angiogenic factor, free IL-12 subunit: p40 is IL-12 antagonist and we aimed to determined its concentration and correlation with angiogenesis induced by sera of diabetic patients.

Materials and methods

18 patients with diabetes mellitus type II, (DM2, 8 with background retinopathy and 10 without retinopathy), 51-90 years old, 11 men and 7 women (mean duration of diabetes 12.1 years, mean HbA1c % 10.2) and 17 with diabetes

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mellitus type I (DM1), all with retinopathy (4 with proliferative retinopathy), 29-68 years old, 10 men and 7 women (mean duration of diabetes 22.4 years, mean HbA1c % 8.3), were enrolled into the study. The control group consisted of 45 non-diabetic people, 32-83 years old, without immunological and inflammatory diseases. From this group, 27 control subjects aged 32-69 years (mean age 53.7±4) served as age-matched control for DM1 patients and 27 control subjects aged 55-83 years (mean age 66.3±2.4) served as age-matched controls for DM2 patients. Forming of two control groups was necessary, because negative correlation between people age and angiogenic activity of their sera was observed [Skopiński et al, in preparation].

The study was approved by the respective Ethics Committees , and written informed consent was obtained from all patients.

Blood samples were allowed to clot for 30 minutes at room temperature before centrifugation for 10 minutes at 1000xg. All samples were stored at –78˚C.

Cytokine concentration

IL-12p40 concentrations were assessed using an enzyme-linked immunoassay with a sensitivity of 5pg/ml (R&D SYSTEMS, Inc. Minneapolis, USA) according to the protocol provided. Briefly: standards and samples were pipetted into the wells pre-coated with a monoclonal antibody specific for IL-12p40, and any IL-12p40 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-12p40 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of IL-12p40 bound in the initial step. The colour development was stopped and the intensity of the colour was measured.

Angiogenesis assay

The angiogenesis assay was performed according to the Sidky and Auerbach experimental model with a number of modifications [4-6]. In brief, 8-week-old, 20 g of body mass, inbred, female Balb/c mice were anaesthetised with 0.2 ml of 3.6% chloral hydrate and both flanks of each mouse were shaved. Four to six samples (0.05 ml) of patient sera were injected intradermally into both lateral flanks of the mouse (three mice per patient). This resulted in 12 to 16 points of neovascularisation induction in each case. The mean value of newly formed blood vessels was determined for each patient. In order to facilitate precise localisation of the injection sites, sera samples were treated with 0.1% trypan blue. After 72 hours the mice were sacrificed with a lethal dose (1 ml) of 3.6% chloral hydrate and their skin was dissected from underlying tissue. Sites of angiogenesis, or blood vessels formed in response to the injection of patient sera, on the inner skin surface, were assessed using a dissection microscope at 6 x magnification. All newly formed blood vessels were identified and counted using the criteria suggested by Sidky and Auerbach [4].

Results

We showed positive correlation ($p<0.05$) between angiogenic activity of DM2 patients sera and their IL12p40 content ($r=0.5625$, Fig. 1).

In case of DM1 patients and sera of people from control group there were no connection between above parameters ($r=0.2659$ and 0.2743, respectively).

The mean number of newly formed blood vessels was significantly higher in DM2 patients as compared to control subjects. However, there were no differences between angiogenic activity in DM2 patients with and without retinopathy (table 1).

Similarly we evaluated higher concentration of IL-12p40 in DM2 patients as compared to control group. No differences between patient with and without retinopathy were detected in IL-12p40 sera concentration (table 1).

In case of DM1 patients angiogenic activity was lower as compared to healthy controls, but IL-12p40 concentration in sera was not statistically different from the control (table 2).

Discussion

Ocular angiogenesis associated with proliferative diabetic retinopathy and age-related macular degeneration is the leading cause of blinding diseases in adults in developed countries. Physiological and pathological retinal neovascularisation may occur independently in postnatal life through the complex activation of pro- and antiangiogenic pathways.

In the present study DM2 patients sera higher angiogenic activity was observed as compared to healthy controls. The differences were observed both in patients with and without retinopathy. It may suggest that higher angiogenic activity in DM2 patients may in consequence lead to microvascular complications. Above observation agrees with our previous

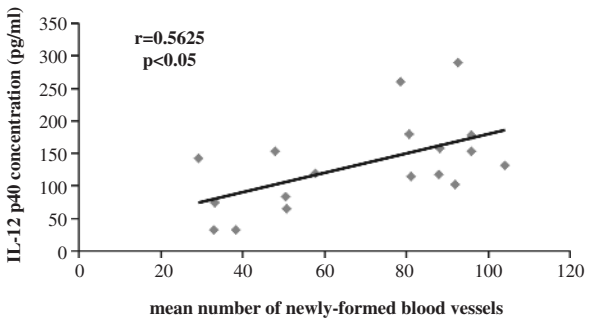

Fig. 1. Positive correlation between angiogenic activity of DM2 patients sera and their IL12 p40 content

Table 1. In vivo angiogenic activity (SIA test) and IL-12 p40 level (ELISA) in DM2 patients and respective age-matched control sera

Source of sera	Mean number of newly-formed blood vessels +/- SE	Mean cytokine concentration (pg/ml) +/- SE
8 DM2 patients with retinopathy (51-79 years old)	*** 66.7±8.3 (96)	** 136±22 (16)
10 DM2 patients without retinopathy (54-90 years old)	*** 75.9±7.8 (120)	* 131±21 (20)
27 controls (without diabetes) (55-83 years old)	44.1±1.4 (324)	74±20 (14)

( ) number of tests in parentheses
* 0.1>p>0.05
** p<0.05
*** p<0.01

Table 2. In vivo angiogenic activity (SIA test) and IL-12p40 level (ELISA) in DM1 patients and respective age- matched control sera

Source of sera	Mean number of newly-formed blood vessels +/- SE	Mean cytokine concentration (pg/ml) +/- SE
17 DM1 patients with retinopathy (29-68 years old)	*** 38.8±6.5 (204)	81±11 (34)
27 controls (without diabetes) (32-69 years old)	58.9±1.3 (324)	61±13 (28)

( ) number of tests in parentheses
*** p<0.01

results showing high angiomodulatory effect of sera from type 2 diabetic patients with background retinopathy [7]. On the contrary in diabetes type 1 patients group the angiogenic acivity was lower than observed in healthy subjects. In our previous studies we evaluated lower angiogenic activity of DM1 patients sera as well as VEGF concentrations that confirm present observations [8].

Higher angiogenic activity may cause high incidence of microvessel complication of diabetes type 2 in study group. However it is widely known that diabetes mellitus type I is connected with proliferative retinopathy much more often than DM type 2.

Taking into consideration that our observation stands in contrary with clinical implications of different types of diabetes we decided to evaluate potential role of angiogenic stimulator: free IL-12 subunit p40 [9].

We showed that in case of diabetes type 2 patients there is higher concentration of IL-12p40 in sera as compared to healthy controls as well as compared to diabetes type 1 patients.

Moreover we evaluated correlation between IL-12p40 sera concentration and angiogenic activity of DM 2 patients sera.

Conclusions arising from above observations: a/ IL-12p40 sub-unit may be partly responsible for high angiogenic activity of DM2 patients sera; b/ more severe retinopathy in DM1 patients group may be partly connected with longer disease duration (22,4 years in DM1 group and 12,1 years in DM2 patients group); c/ it seems to be very important to find potential inhibitor of microvessels development present in sera of DM 2 but not DM 1 patients that possibly prevents retinopathy development. It is probable that inhibitors of proteases play the significant role in above process [10].

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