Important role of bFGF in the angiogenic activity of human serum evaluated by the mouse cutaneous test

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Abstract

Basic fibroblast growth factor (bFGF) is one of the key and most extensively investigated angiogenic cytokine. Its activity has been evaluated in different in vitro as well as in vivo experimental models. We have previously shown, that human serum but also human bFGF induced significant neovascular response in the mice cutaneous assay. The aim of the present study was to confirm the direct proangiogenic effect of human bFGF in the mice in vivo model. Therefore, the effect of human serum absorption with anti-bFGF antibody on its angiogenic activity in serum-induced mice angiogenesis test (SIA) has been evaluated. As a result, significantly diminished angiogenic activity of sera preincubated with the anti-bFGF antibody has been demonstrated, with negligible effect of the normal goat IgG serving as a control. Consequently, the significant direct role of human bFGF in the neovascularization induced by human serum in the mice skin has been proven. Present study clearly demonstrated as well that mice cutaneous assay might serve as the in vivo model for monitoring of the angiogenic effect of human bFGF.

Key words: *bFGF, angiogenesis, human serum, mice.*

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Introduction

Substantial number of growth factors has been found to play significant role in the angiogenesis processes. Among them, basic fibroblast growth factor (bFGF) remains one of the most extensively investigated angiogenic cytokine. bFGF is a heparin-binding protein and shows considerable angiogenic activity in different experimental models [1]. It was demonstrated to stimulate *in vitro* endothelial cell proliferation and sprouting. Recombinant human bFGF was proven to produce strong angiogenic reaction *in vivo* in mice following its intradermal injection [2]. In experimental dogs, bFGF administered intramyocardially increased the growth of microvessels and improved the left ventricular function in acute myocardial infarction [3].

In addition to multiple tissue sources, significant pool of bFGF is also present in blood serum [4]. We have shown previously, that human serum induced neovascular response when introduced intradermally into the mice skin [5-9]. The aim of the present study was to confirm the direct proangiogenic effect of human bFGF in the mice *in vivo* model by determining the effect of human serum absorption with anti-bFGF antibody on its angiogenic activity in serum-induced mice angiogenesis test (SIA).

Material and methods

Serum samples were collected from four healthy subjects, aged 46-52 years. Serum was isolated by centrifugation, aliquoted and stored at –78˚C until used.

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Table 1. Mean number of newly-formed blood vessels induced by human sera in mice skin following neutralization with anti-bFGF antibodies

	Incubation with normal goat IgG (control)		Incubation with anti-bFGF antibodies		
serum donor initials	**n**	**mean ±SE**	**n**	**mean ±SE**	**difference**
WJ	12	17.1±1.51	17	11.8±0.69	p<0.01
SA	15	15.2±0.78	18	7.7±0.41	p<0.001
KJ	15	13.5±0.65	14	10.4±0.74	p<0.01
ZR	17	21±1.21	13	9.4±0.81	p<0.001
Total	**59**	**16.7±0.36**	**62**	**9.8±0.18**	**p<0.01**

n – number of injections.

Informed consent for blood drawing was obtained from each participant according to the institutional review board--approved protocol. The study was approved by a local Ethical Committee.

Neutralization of bFGF with anti-human bFGF antibodies

Absorption was performed using polyclonal goat anti--human bFGF antibodies and goat IgG as a control (R&D Systems, USA). Neutralizing concentration of anti-bFGF antibody was chosen according to the neutralization curve provided by the manufacturer. The reagents resuspended in PBS were mixed with respective serum samples to obtain the final concentration of 10 µg/ml and incubated for 60 min at 37°C and 5% CO_2 atmosphere.

Mice cutaneous angiogenesis assay

The cutaneous angiogenesis assay (serum-induced angiogenesis, SIA) was performed as previously described [5-9]. Briefly, study was performed in 2-month old, female inbred Balb/c mice. Mice have been of local laboratory breed, weighing ca 20 g each. Serum was injected intradermally (0.05 ml per one injection, 4-6 injections per mouse, at least 3 mice for each tested serum sample) into regionally shaved, anaesthetized with chloral hydrate (POCH, Poland) Balb/c mice. In order to facilitate the localization of injection sites, the serum samples were dyed with 0.1% of trypan blue.

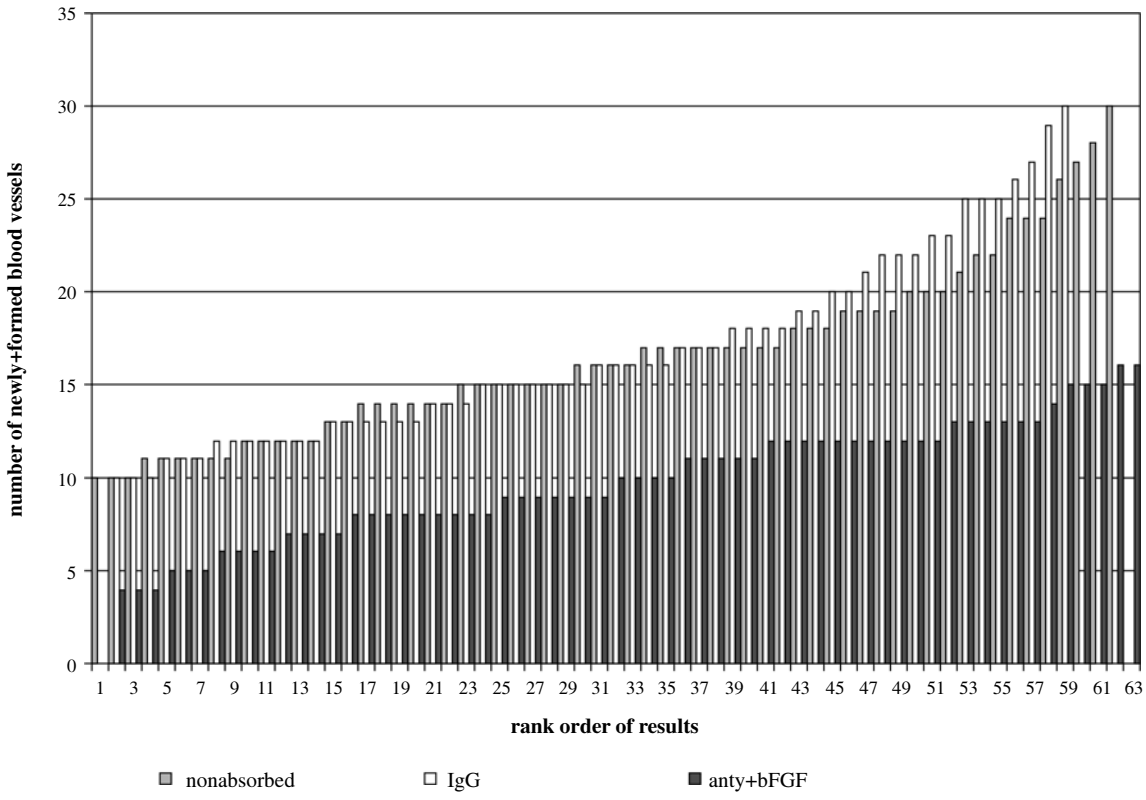

Fig. 1. The effect of preincubation with anti-bFGF antibody on the angiogenic activity of human sera in the SIA test

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, at 6 x magnification, in 1/3 central area of microscopic field. Identification was based on standard criteria (newly-formed blood vessels differ from background vasculature by their small size, tortuosity and divarications), as previously described.

All experiments were approved by the local Ethical Committee.

Measurement of bFGF concentration

Cytokine concentration in examined sera samples was determined by sandwich ELISA method (R&D Systems, USA), using ready-to-use kits for human bFGF (high sensitivity) according to the manufacturer instructions. Optical density was measured at 450 nm using spectrophotometric reader Elx800 (Biotek Instruments, Inc., USA). Cytokine concentration was expressed as pg/ml.

Statistical analyses were performed by Student *t*-test.

Results

Results are presented in table 1 and figure 1 (results for each individual serum shown separately). The angiogenic activity of all examined sera was significantly diminished following neutralization with anti-bFGF antibodies. Meanwhile, the effect of incubation with normal goat IgG on the angiogenic activity in SIA test was almost negligible. Mean bFGF concentration measured before absorption was 19.2 pg/ml, while in samples incubated with control IgG and with anti-bFGF antibodies, respectively 12.6 pg/ml and 3.7 pg/ml.

Disscusion

Basic fibroblast growth factor was one of the first identified angiogenic growth factors. It is expressed by multiple cell types and is important in morphogenesis, development hematopoiesis and tumorogenesis. bFGF is a heparin-binding protein tightly associated with the extracellular matrix and can be released as a bioactive bFGF-glycosaminoglycan complex [10-12]. It has been proven that bFGF-induced neovascularization requires signalling through specific receptors, but also junctional adhesion molecule 1 (JAM1) and alphavbeta3 integrin [13]. Though significant cross-reactivity of anti-human bFGF antibodies with mouse bFGF is a well known fact, our previous data demonstrating that recombinant human bFGF produced strong angiogenic reaction in mice cutaneous assay were noteworthy [2]. Subsequently, we have shown that intradermal injection of human serum also induced considerable neovascular response in mice [5-9]. Present study has confirmed this phenomenon and clearly showed that human bFGF plays significant role in the new-blood vessels formation induced by human serum in mice skin. Moreover, serum absorption test demonstrated significantly decreased serum angiogenic activity following bFGF neutralization with specific antibodies and proved therefore its direct interaction with specific receptors in host tissue.

Above observations confirm that mice cutaneous assay might serve as the *in vivo* model for monitoring of the angiogenic effect of human bFGF but also other biological materials containing (serum) or actively producing (lymphocytes, cancer cells) this cytokine. Similarly, mice cutaneous assay might serve as the eligible experimental setup for the evaluation/screening of the potential anti- or proangiogenic effect of biologically active substances.

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