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Genetic Modification of Adipose Derived Stem Cells with Recombinant Plasmid DNA pBud-VEGF-FGF2 Results in Increased of IL-8 and MCP-1 Secretion

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Bacterial plasmid DNA is often used in gene therapy application as a vector for delivering recombinant genes into eukaryotic cells. In this work we study effect of plasmid DNA encoding pro-angiogenic growth factors VEGF and FGF2 on cytokine production of human adipose derived stem cells (ADSC). Adipose tissue contain progenitor and stem cells with angiogenic potential which are considered promising cell source for treating ischemic conditions. Genetic modification of human ADSC with recombinant plasmids encoding therapeutic growth factors might change therapeutic potential of these cells. We report that genetic modification of ADSC with recombinant plasmid pBud-VEGF-FGF2 results in expected increased of VEGF secretion and also results in increase of IL-8 and MCP-1 secretion into culture medium.

Key words: Bacterial plasmid DNA, Cytokines, Chemokines, Stem cells, Stromal vascular fraction, Vascular endothelial growth factor, Basic fibroblast growth factor, Genetic modification.

Mesenchymal stem cells (MSCs) are one of the most promising tools for cell-based gene therapy due to their ability to multi lineage differentiation, simplicity of isolation, as well as their high *ex vivo* expansion potential. Fat tissue is rich source of mesenchymal stem cells. It has been shown that cells derived from adipose tissue are one of the most safe, easily accessible in preparative quantities and have high regenerative potential[1]. Adipose derived stem cells (ADSCs) have many clinical implications. For example, lipofilling with autologous fat tissue enriched by stromal-vascular fraction cells used for treatment of the idiopathic progressive hemifacial atrophy[2].

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Stem cells from adipose tissue (Adipose Derived Stem Cells, ADSCs) secrete variety of trophic and protective factors including VEGF. The basic fibroblast growth factor (FGF2) and vascular endothelial growth factor (VEGF) are potent inducers of angiogenesis and lymphangiogenesis *in vivo* and *in vitro*. It has been shown that direct injection of plasmid pBud-VEGF-FGF2 in the proximal and distal segments of nerve, as well as in the auto-nerve graft, stimulates the regeneration of the rat's sciatic nerve and restores motor activity, partially through increase in angiogenesis in transplanted tissue[3]. Clinical studies have shown that intramuscular injection of plasmid, encoding for VEGF and FGF2, results in successful treatment of distal form of peripheral artery disease[4].

Genetic modification of the ADSCs using VEGF, FGF2 and other therapeutic growth factors can significantly improve their therapeutic potential. It has been shown that umbilical cord

blood cells (UCBC) over expressing growth factors are effective for treatment of neurodegenerative diseases[5]. However, despite therapeutic efficacy, little is known about the effect of genetic modification of ADSCs on cytokine secretion. Here we present analysis of cytokines and chemokine secretion by stem cells from human adipose tissue, genetically modified using pBud-VEGF-FGF2 plasmid.

MATERIALS AND METHODS

Human adipose derived stem cells isolation. ADSCs were isolated as previously described with few modifications[6]. Samples of subcutaneous adipose tissue were obtained during routine cosmetic surgery (liposuction). In the sterile laminar box, adipose tissue was washed extensively with equal volumes of PBS. Then the tissue was dissociated with 0,2% solution of collagenase (Biolot, Russia), for 45 min at 37°C with intermittent shaking. Digested tissue was centrifuged for 5 min at 1400 rpm at room temperature. The supernatant, containing mature adipocytes, was aspirated and the pellet was identified as the stromal vascular fraction (SVF). SVF was resuspended in the Dulbecco's modified Eagle's medium (DMEM, PanEco, Russia) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine. Finally, these cells were incubated overnight at 37°C in a humidified atmosphere, 5% CO_2. After incubation for 24 hours, medium was changed to remove non-adherent cells and cells were incubated for 5-7 days until monolayer of cells was formed (75-90% confluent). Cell morphology was analyzed using an AxyObserver.Z1 inverted microscope (CarlZeiss, Germany) running AxyoVision Rel. 4.8 software.

Human Embryonic Kidney 293 (HEK293) cells were purchased from ATCC. HEK293 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin, 2 mM L-glutamine.

Flow cytometry characterization. ADSCs cell surface antigen phenotype was analyzed using flow cytometry as previously described[7]. Following cell-surface epitopes were marked with anti-human antibodies: CD14, CD45, CD90, CD105, CD133, CD166 (SantaCruz Biotechnology Inc., SantaCruz, CA, USA), CD29-phycoerythrin (PE) (BD Biosciences; San Diego, CA, USA), CD73 (Zymed, SanFrancisco, CA, USA). After incubation with antibody against cell surface marker, cells were washed and incubated with FITC-conjugated secondary antibodies. Cellular events (5000-10000) were acquired and analyzed by flow cytometry using FACScalibur cytometer (Becton Dickinson, SanJose, CA, USA).

Bacterial plasmid DNA preparation. Stocks of enhanced green fluorescent protein (EGPF)-encoding plasmid pEGFP-N2 (ClonTech, Mountain View, CA, USA) and pBud-VEGF-FGF2 plasmid were prepared in Top10 *Escherichia coli* (Invitrogen, USA) using the endotoxin-free Maxi Purification Kit (Qiagen GmbH, Hilden, Germany). DNA concentration was adjusted to 1 mg/mL in Tris-EDTA, and plasmids were stored at -20°C. The pBud-VEGF-FGF2 plasmid was designed based on pBudCE4.1 (Invitrogen, USA) mammalian expression vector. Into two multiple cloning sites of this vector have been inserted vascular endothelial growth factor (VEGF, isoform 165) and basic fibroblast growth factor (FGF2) genes. The pBudCE4.1 vector can simultaneously and independently express two recombinant genes under the control of the strong elongation factor EF1alpha and cytomegalovirus CMV promoters[8]. Purification of plasmid DNA from recombinant strains of *E. coli* was performed using PlasmidSelect Xtra Starter Kit (GE Healthcare) according to manufacturer instructions as described previously[9].

Genetic modification of adipose derived stem cells and HEK293. Variety of methods, including physical (electroporation, sonycation, gene gun, etc.), chemical (lipoplexes, polyplexes, cationic nanoparticles, etc.), can be used for genetic modification (transfection) of ADSCs[10]. Cell transfection was carried out using TurboFect *in vitro* Transfection Reagent (Thermo Scientific Inc., USA) according to the manufacturer guidelines. Expression of EGFP in transfected cells was examined at 48 h post transfection using fluorescence microscopy.

Collection of conditioned medium. Conditioned media was collected 24 h after transfection, centrifuged at 600 g for 5 min and filtered a 0,22 µm filter. Cell supernatants were stored at -80°C until analyzed. For multiplex and

ELISA assays, each sample was diluted 10 times.

ELISA assay. VEGF concentration in conditioned media was determined using VEGF ELISA kit (A-8784, Vector, Russia) following the manufacturer's protocols.

Cytokine and Chemokine Measurement by Multiplex Bead Array Kits. Culture supernatant samples were thawed only once before performing the MILLIPLEX® MAP Human Cytokine/ Chemokine Panel (Merck Millipore, Billerica, MA), a bead-based multiplex immunoassay, which allows the simultaneous quantification of the following 7 human cytokines: INF-γ (Interferon gamma, cat. #MXHIFNG, Bead 20), IL-1β (Interleukin-1 beta, cat. #HSIL-1B, Bead 24), IL-2 (cat. #MXHIL-2 Bead 28), IL-8 (cat. #MXHIL-8, Bead 40), IL-10 (cat. #MXHIL-10, Bead 44), IL-12 (cat. #MXH12P70, Bead 48), MCP-1 (Monocyte chemotactic protein type 1, cat. #MXHMCP-1, Bead 58) (Merck Millipore, Billerica, MA). Culture supernatant samples were processed following the manufacturer's protocols. Cytokine analyzes was performed using Luminex® 200™ detection system (Merck Millipore, Billerica, MA) with Luminex IS 2.3 software using standard curves.

Statistical analysis. For each set of experiments, values were reported as means ± standard deviation (SD). The results were evaluated by using Student's -test. All statistical analyses were performed with Microsoft Excel 2007 software package. Values were considered significant when <0,05.

RESULTS AND DISCUSSION

Human adipose tissue was utilized for isolation and culture of ADSCs. Morphological and phenotypic characteristics of ADSCs were similar to that of human mesenchymal stem cells (MSCs). After three consecutive passages, ADSCs displayed typical fibroblastoid morphology. The ADSCs were capable of long-term proliferation *in vitro* (7–8 passages), and have the capacity to differentiate into orthodox ways: adipogenic, osteogenic and chondrogenic (data not shown). Using flow cytometry analysis, we established that the most of the third passage ADSCs express the surface antigens (CD markers) typical for human MSCs: CD29, CD73, CD90, CD105 and CD166, and lack hematopoietic cell surface antigens CD14, CD45 and CD133 (data not shown).

One of the strategies for improving the therapeutic capacity of transplanted cells is their genetic modification to increase secretion of therapeutic factors or to limit the adaptive differentiation[6]. Here we present our data transfection of ADSCs with plasmid pBud-VEGF-FGF2 containing open reading frame (ORF) for VEGF and FGF2. To evaluate the efficacy of transfection, ADSCs were cotransfected with pBud-VEGF-FGF2 pEGFP-N2 plasmids. pEGFP-N2 plasmid contains ORF for green fluorescent protein (GFP). Therefore, expression of GFP was followed to evaluate the efficacy of pBud-VEGF-FGF1-N2 transfection Approximately 50–60% of genetically modified cells expressed EGFP by the day 14 after transfection (Fig. 1).

ELISA assay of ADSCs and HEK293 cells conditioned media showed that level of VEGF production by cells transfected with pBud-VEGF-FGF2 plasmid was higher than the non-transfected cells and cells transfected pEGFP-N2 plasmid (Fig. 2).

A comparative analysis of cytokine production by native and genetically modified ADSCs has been performed. Cytokines analyzed include proinflammatory Th1-cytokines (INF-γ, IL-1β, IL-2, IL-12), Th2 cytokine IL-10 and chemokines, IL-8, MCP-1. Increased production of IL-8 and MCP-1 has been detected in ADSCs transfected

Table 1. Multiplex quantitative analysis of cytokines and chemokines secreted by genetically modified and native ADSCs. pBud-VEGF-FGF2, pEGFP-N2 — supernatants from transfected cells, NTC — supernatant from non-transfected cells

Modification	Cytokines/chemokines concentration (pg/mL)						
	IFN-γ	IL-1β	IL-2	IL-8	IL-10	IL-12	MCP-1
pBud-VEGF-FGF2	5	5	3	24918	21	10	26858,5
pEGFP-N2	2	5	4	2268	15	8	3702
NTC	5	4	3	1588	12	8	4954,5

with pBud-VEGF-FGF2 as compared to non-transfected cells and cells transfected pEGFP-N2 plasmid (Table 1).

IL-8 is a chemokine produced by macrophages, epithelial and endothelial cells. IL-8 is known to be a potent inducer of angiogenesis[11] and neutrophil chemotactic factor. It has been shown that the addition of recombinant IL-8 to HUVEC cells results in increased cells survival, proliferation and production of matrix metalloproteinase MMP-2 and MMP-9[12].

Monocyte Chemoattractant Protein 1 (MCP-1/CCL2) is a member of the C-C chemokine family, and a potent chemotactic factor for monocytes, memory T cells, and dendritic cells. MCP-1 recruits cells to the sites of inflammation produced by either tissue injury or infection. Platelet derived growth factor (PDGF) is a basic inducer of *CCL2* gene. MCP-1 is expressed in vascular endothelial cells in the arterial wall. It is known that CCL2-induced activation of the CCR2 receptor on these cells is responsible for regeneration of endothelium after injury[13], angiogenesis and collateral vessel formation *in vivo*[14-15]. It has been shown that the CCL2-induced angiogenesis is mediated through pathways involving VEGF and activation of RhoA small G protein (RhoA)[16]. This may explain upregulation of MCP-1 levels in conditioned medium of pBud-VEGF-FGF2 genetically modified ADSCs.

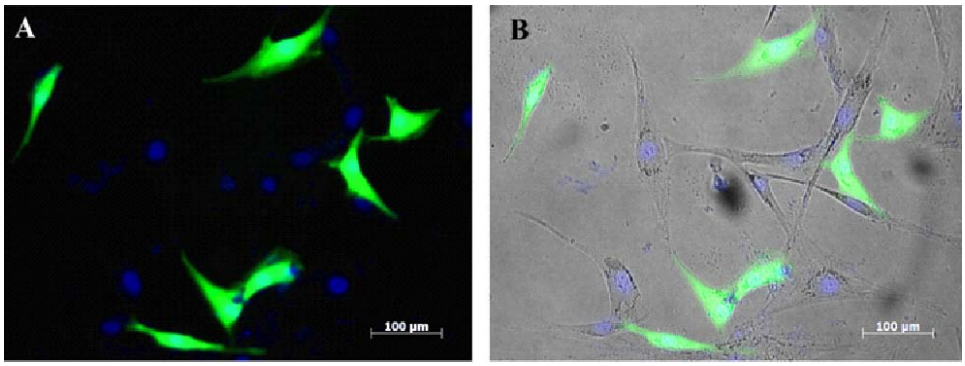

Fig. 1. Fluorescent microscopy of human ADSCs. ADSCs were transfected with pEGFP-N2 vector, expressing the gene of the enhanced green fluorescent protein EGFP (green). Nuclei were stained with DAPI (blue). A — fluorescence microscopy. B — the phase-contrast microscopy with merged image from slide A. Scale bar, 100 µm.

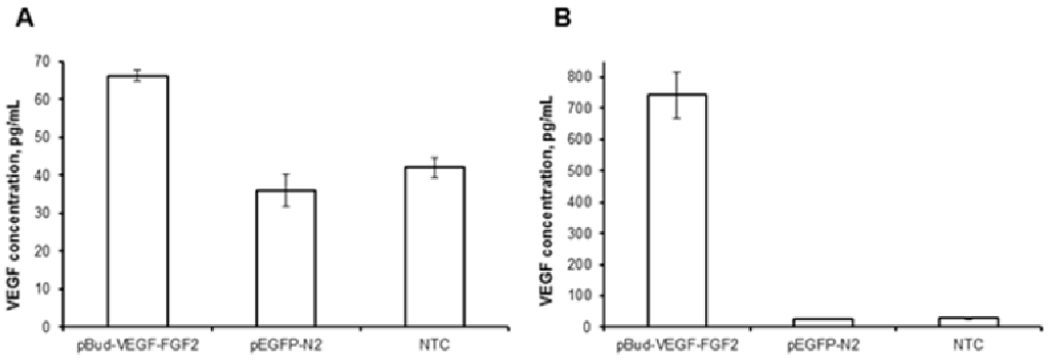

Fig. 2. ELISA analysis of VEGF concentration supernatant of ADSCs (A) and HEK293 (B). Supernatants were collected at 24 h after transfection with pBud-VEGF-FGF2 or pEGFP-N2 plasmids. pBud-VEGF-FGF2, pEGFP-N_2 — supernatants from transfected cells. NTC — supernatant from non-transfected cells. Data is presented as average ± SD (n=3).

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