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Human Adipose Derived Stem Cells Do Not Alter Cytokine Secretion in Response To The Genetic Modification With pEGFP-N2 Plasmid DNA

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Abstract: Adipose tissue contain progenitor cells with regenerative (angiogenic, neuroprotection, trophic etc.) potential and treatment based on adipose tissue-derived stem cells (ADSCs) transplantation may constitute a promising therapy. Genetic modification of stem cells with genes encoding growth factors and other biologically active molecules might further increase therapeutic efficiency. However, there is a question about the advantages and disadvantages of such gene/stem cell therapy. For a better understanding of the mechanisms occurring in organism after cell transplantation we should first study cell behavior *in vitro*. Here we report results of ADSCs transfection with plasmid DNA pEGFP-N2 and subsequent study of cytokines and chemokines secretion using Luminex technology. We demonstrated that genetic modification of ADSCs with commonly used control plasmid vector, encoding enhanced green fluorescent protein (EGFP), did not affect secretion of cytokines/chemokines IFN-γ, IL-1β, IL-2, IL-8, IL-10, IL-12, MCP-1. Thus, genetic modification procedure on its own might have little effect on stem cell properties, making it useful for gene-stem cell therapy applications.

Key words: Plasmid · pEGFP-N2 · Cytokines · Chemokines · Human Adipose Derived Stem Cells · Transfection

INTRODUCTION

One of the most promising sources of stem cells is the fatty tissue. Stem cells from adipose tissue (Adipose tissue-Derived Stem Cells, ADSC) secrete a variety of trophic and protective factors. It might explain some of therapeutic efficacy after transplantation [1]. Genetic modification of stem cells with genes encoding growth factors and other biologically active molecules might further increase therapeutic efficiency. However, there is a question about the advantages and disadvantages of such gene/stem cell therapy. In this paper we report our results of ADSCs transfection with plasmid DNA pEGFP-N2 (BD Biosciences Clontech, Germany) and subsequent study of cytokines and chemokines secretion using Luminex technology.

MATERIALS AND METHODS

Isolation and Culture of Human Adipose Derived Stem Cells: Stem cells from adipose tissue (Adipose Derived Stem Cells, ADSCs) were isolated as previously described with minor modifications [2-3]. Samples of subcutaneous adipose tissue were obtained during a routine cosmetic surgery (liposuction). Within 30-40 minutes tissue after liposuction has been delivered to the laboratory. In biosafety cabinet lipoaspirate was wash of from blood. The resulting homogeneous lipoaspirate was treated with 0.2% crab collagenase solution (159 PE/mg Biolot, Russia). Cells were spun down and erythrocytes were removed using hypotonic cell lysis buffer (0,168 M NH_4Cl, 0,1 M $KHCO_3$, 1,27 mM EDTA, pH 7,3). Cells were cultured in áMEM medium, supplemented with 10% fetal

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bovine serum, 2 mM L- glutamine, 1% antibiotic mixture of penicillin and streptomycin (PanEco, Russia) at 37 °C in a humidified atmosphere of 5% CO_2. After 24 hours medium was replaced with fresh [4]. After 5-7 days of incubation was observed the formation of a monolayer of cells. Subsequent passaging of cells and medium replacement was carried out according to standard protocols. Cell morphology was evaluated using inverted microscope AxyObserver.Z1 (CarlZeiss, Germany) using software AxyoVision Rel. 4.8. [5-6].

Phenotyping of Cells: Surface antigens of ADSCs were investigated using flow cytometry as previously described [7]. Cells were trypsinized, washed with PBS and incubated with primary antibodies against: CD14 (cat #SC-7328), CD29 (cat #BD556049), CD45 (cat #SC-70686), CD90 (cat #SC-53456) CD105 (cat #SC-71043), CD133 (cat #SC-65278), CD166 (cat #SC-53551) (SantaCruz Biotechnology Inc., SantaCruz, CA, USA) CD73 (cat #550256) (Zymed, SanFrancisco, CA, USA). The cells were then washed of excess primary antibody with PBS and incubated with secondary antibodies conjugated with FITC (Fluorescein isothiocyanate) except for CD29, conjugated with PE (R-Phycoerythrin). Flow cytometry was performed on cytometer FACScalibur Becton Dickinson, SanJose, CA, USA) during which 5000-10000 events were evaluated for each experiment.

Transfection of Cells and Collection of Conditioned Medium: Plasmid DNA pEGFP-N2 was isolated using QIAfilter Plasmid Midi Purification Kit (QIAGEN, USA) according to manufacturer instructions.

A variety of methods, such as physical (electroporation, sonication, gene gun, etc.), chemical (lipoplexes, polyplexes, cationic nanoparticles, etc.), can be used for transfection of ADSCs [8]. Transfection of ADSCs with a plasmid vector pEGFP-N2 (BD Biosciences Clontech, Germany), expressing enhanced green fluorescent protein EGFP, was carried out using transfection reagent TurboFect (Thermo Scientific Inc., USA) according to the procedure recommended by the manufacturer as previously described [9]. The transfection efficiency was determined by the number of EGFP positive cells by fluorescence microscopy on an inverted fluorescence microscope AxioOberver.Z1 (Carl Zeiss, Germany). The culture medium from cells transfected with plasmids were harvested 24 hours after transfection and was purified from residual cellular material by centrifugation and filtration through 0.45 micron nylon filter. For multiplex analysis, each test sample was diluted 10 times.

Multiplex Analysis of Cytokines: Cytokine analysis was performed using commercially available fluorophore conjugated microspheres (fluorophore-conjugated bead) MILLIPLEX ® MAP, in accordance with the instructions of the manufacturer (Millipore). Soluble cytokine molecules in the supernatant was assessed using the composite panel comprising of the following analytes: 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) (Millipore), in which the concentration of standards ranged from 3,2 to 10,000 pg/ml. Each test sample was diluted 10 times prior to analysis. Samples were incubated with antibodies conjugated to microspheres. Complexes were washed twice and incubated with biotinylated secondary antibodies. For visualization of immune complexes was incubated streptavidin conjugated PE and detected using Luminex® 200™ detection system (Millipore). Analyte concentration was calculated using a standard curve with software Luminex IS 2.3, provided by the equipment manufacturer.

RESULTS AND DISCUSSION

In our study we demonstrated that human adipose derived stem cells, according to their morphological and phenotypic characteristics, were similar to human mesenchymal stem cells (MSCs). The cells had fibroblast-like morphology, were capable of long-term proliferation *in vitro* (7-8 passages) and had the capacity to differentiate into adipogenic, osteogenic and chondrogenic lineages [10] (data not shown).

It was established by flow cytometry that most of the third passage cells were expressing surface antigens (differentiation markers), characteristic to human MSCs: CD29, CD73, CD90, CD105 and CD166. The differentiation markers CD14, CD45 and CD133, characteristic to hematopoietic cells, showed low level of expression (Figure 1).

One of the possible strategies for improving the therapeutic capacity of transplanted cells is their genetic modification to enhance secretion of therapeutic factors or to influence cell differentiation. In particular for this purpose one may use both viral and non-viral gene delivery systems [11-12]. In the present study, we transfected ADSCs with plasmid vector pEGFP-N2 with a commercially available transfection reagent TurboFect

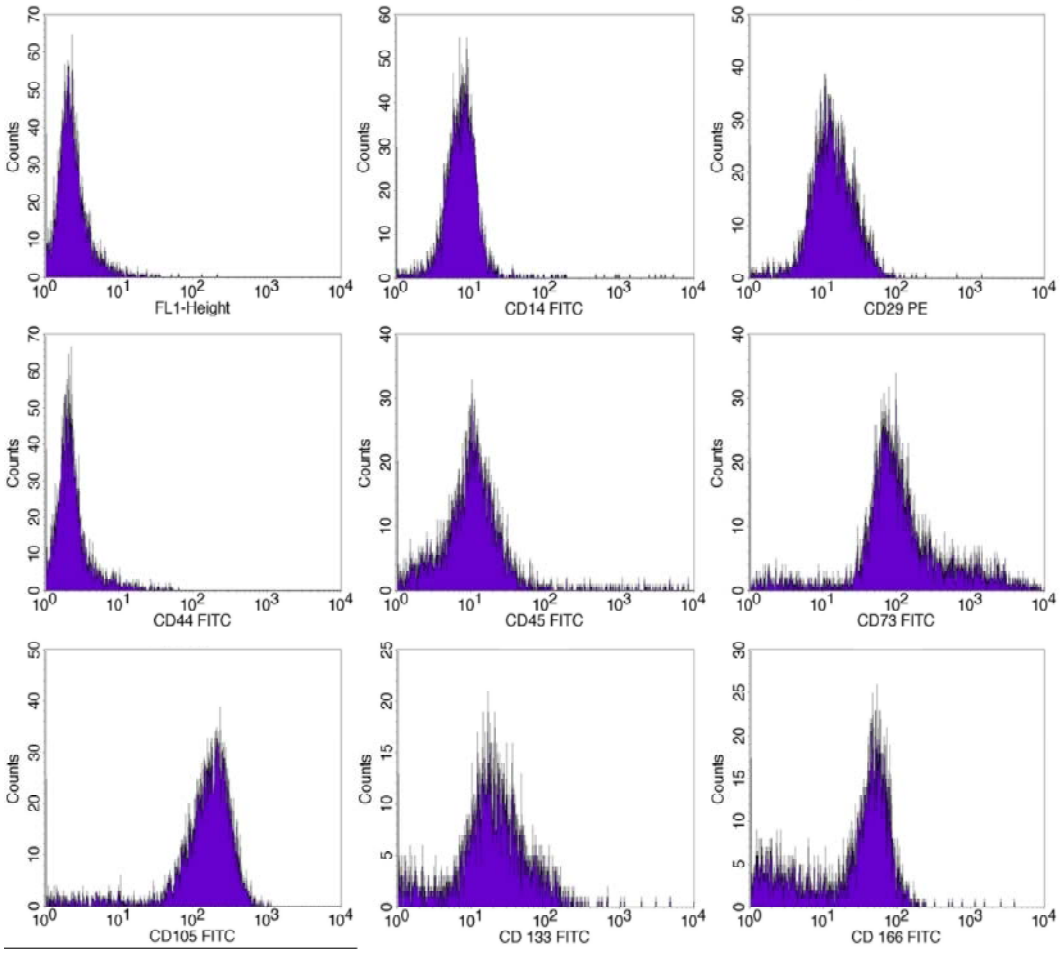

Fig. 1: Characterization of surface antigens of human adipose derived stem cells (ADSC) using flow cytometry. Antibodies to hematopoietic cells markers: CD 14, CD45, CD133; mesenchymal stem cell markers: CD29, CD73, CD90, CD105 and CD166. Flt1-Height - control (negative cells), FITC - fluorescein isothyocyanate, PE – phycoerythrin.

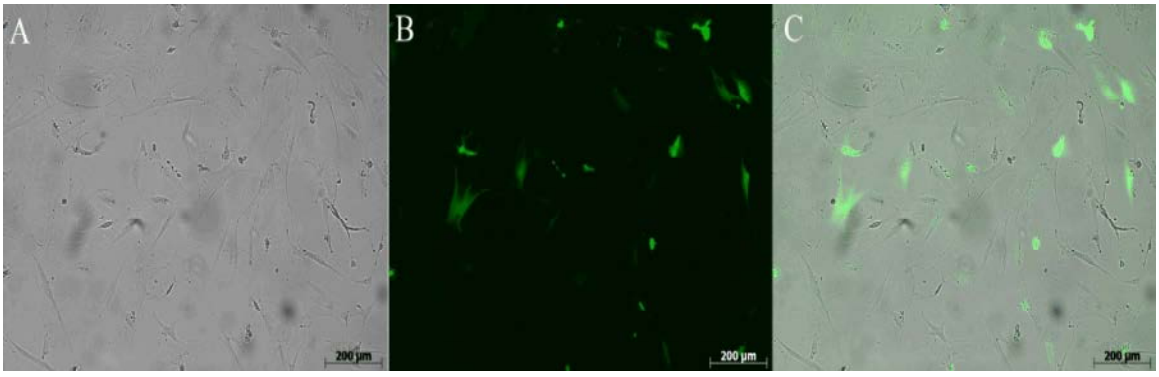

Fig. 2: Fluorescent analysis of human adipose derived stem cells (ADSCs), 48 hours after transfection with plasmid pEGFP-N2, expressing enhanced green fluorescent protein EGFP. A - phase-contrast microscopy; B - fluorescence microscopy. C - merged image from slides A and B. The scale bar 200 mkm.

Table 1: Multiplex quantitative analysis of the secreted cytokines and chemokines in native and genetically modified human adipose derived stem cells (ADSCs). pEGFP-N2 - supernatant from transfected cells, NTC - supernatant from non-transfected cells

	cytokines / chemokines (pg/ml)						
Supernatant	IFN-γ	IL-1β	IL-2	IL-8	IL-10	IL-12	MCP-1
pEGFP-N2	2	5	4	2268	5	8	3702
NTC	5	4	3	1588	2	8	4954,5

(Thermo Scientific Inc., USA). Transfection efficiency was assessed by expression of the recombinant protein EGFP using fluorescence microscopy (Figure 2). Visually, about 50-60% of cells expressed EGFP after transfection.

A comparative analysis of native and genetically modified ADSCs showed low level production of IFN-γ, IL-1β, IL-2, IL-10, IL-12 while increased production of IL-8 and MCP-1 (Table 1). No statistically significant differences were observed between original and genetically modified ADSCs.

Thus, we demonstrated that genetic modification of ADSCs with commonly used control plasmid vector, encoding enhanced green fluorescent protein (EGFP), did not affect secretion of cytokines/chemokines IFN-γ, IL-1β, IL-2, IL-8, IL-10, IL-12, MCP-1. Thus, genetic modification procedure on its own might have little effect on stem cell properties, making it useful for gene-stem cell therapy applications.

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