Adipose-derived stem cells secrete neurotrophic factors

L Clauser, R Tieghi, A Palmieri, F Carinci

Abstract

Introduction
Structural fat grafting is a widely approved technique in craniomaxillofacial reconstructive surgery used to treat several types of diseases including congenital and post-traumatic deformities, and to fill out tissue depression due to orbital and periorbital surgery, scars or cancer resections.

Adipose tissue is considered a secreting organ that produces special proteins such as adipokine and neurotrophic factors such as nerve growth factor and brain-derived neurotrophic factor.

Given their role in stimulating repair of peripheral nerves, their expression could influence the good outcome of a structural fat grafting. This study discusses the ability of adipose-derived stem cells to secrete neurotrophic factors.

Materials and methods
In this study, we compared the ability of adipose-derived stem cells and adipocytes, obtained from the same patient, to secrete these two neurotrophic factors. The expression levels of nerve growth factor and brain-derived neurotrophic factor were measured using quantitative real-time polymerase chain reaction.

Results
Stem cells showed up-regulation of brain-derived neurotrophic factor and nerve growth factor with respect to adipocytes.

Discussion
These result demonstrated that adipose tissue is an abundant and easily accessible wealth of mesenchymal stem cells and that these cells are responsible for the production of nerve growth factor and brain-derived neurotrophic factor, which are involved in tissue and nerve repair.

Conclusion
Adipose tissue is easily obtainable by lipoaspiration with minimal discomfort to the patient, and its MSC content is adequate for clinical-grade cell manipulation in regenerative medicine.

Introduction

Structural fat grafting is a widely approved technique in craniomaxillofacial reconstructive surgery. It can be used to treat several types of diseases including congenital and post-traumatic deformities, and to fill out tissue depression due to orbital and periorbital surgery, scars or cancer resections.

To restore the normal shape and volume of soft tissue, regenerative medicine exploits a natural resource that is human adipose tissue. For years, several methods followed one another; but the one introduced by Coleman in 1991 is still successfully employed. Briefly, it provides the aspiration, purification and subsequent reinjection of autologous fat. Each step must be performed carefully to prevent fatty structure damage. The importance and usefulness of this approach are due to its high yield in terms of survival rate of fat grafting (of up to 90%).

Adipose tissue is an abundant and easily accessible wealth of mesenchymal stem cells (MSC), which are able to differentiate into different types of cell lines, thus restoring soft tissues. It is considered a secreting organ which produces special proteins such as adipokine and neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF).

NGF is a potenti-diabetic and anorexigenic factor, with an important role in differentiation and plasticity of the central nervous system, body weight control and energetic homeostasis.

Given their role in stimulating repair of peripheral nerves, their expression could influence the good outcome of a structural fat grafting.

In this study we compared the ability of adipose-derived stem cells (ADSCs) and adipocytes, derived from the same patient, to secrete these two neurotrophic factors. The expression levels of NGF and BDNF were measured using the quantitative real-time polymerase chain reaction (qRT-PCR).

Materials and methods

This study conforms to the values laid down in the Declaration of Helsinki (1964). The protocol of this study has been approved by the relevant ethical committee related to our institution in which it was performed. All subjects gave full informed consent to participate in this study.

Stem isolation
Human adipose tissue was obtained by liposuction of adult volunteer

License OA Publishing London 2013. Creative Commons Attribution License (CC-BY)

patients. Fat was finely minced with sterile scissors and transferred to a tube containing digestive solution (DMEM containing 1 mg/ml of collagenase type II). The tube was placed in 37°C water bath for 60 min, swirling occasionally.

The sample was centrifuged at 1300 rpm for 5 min. Then, it was removed from centrifuge, shaken vigorously (to complete separation of stromal cells from primary adipocytes) and centrifuged again for 5 min. The oil on the top of the tube (which includes primary adipocytes) was aspirated and discarded, while the stromal fraction at the bottom was washed three times with 10 ml of PBSA 1× and centrifuged again for 5 min. After the last wash, the pellet was resuspended in 10 ml of Alphamem medium (Sigma Aldrich, Inc., St. Louis, MO), supplemented with 10% foetal calf serum, antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml—Sigma, Chemical Co., St. Louis, MO) and amino acids (l-glutamine—Sigma, Chemical Co., St. Louis, MO). The medium was changed after 2–3 days. Cells were characterized for staminality by flow cytometric analyses.

**Flow cytometric analyses**

The purity of cell cultures was determined by analysis of different antigens after staining with fluorochrome (FITC- or PE-) conjugated mAbs anti-human CD14-FITC, CD14-PE, CD34-FITC, CD45-FITC, CD90-PE, CD105-PE (Immunotech, Marseille, France), and analysed by FACScan. The non-specific mouse IgG was used as isotype control (Immunotech). To avoid non-specific fluorescence from dead cells, live cells were gated tightly using forward and side scatter (Figure 1).

**Primary human dermal fibroblast (HFB) cells culture**

Figments of dermal tissue of healthy volunteers were collected during operation. The pieces were transferred to 75 cm² culture flasks containing DMEM medium (Sigma Aldrich, Inc., St. Louis, MO) supplemented with 20% foetal calf serum and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml—Sigma Aldrich, Inc., St Louis, MO).

Cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. Medium was changed the next day and twice a week. After 15 days, the pieces of dermal tissue were removed from the culture flask. Cells were harvested after 24 days of incubation.

**RNA processing**

Total RNA was isolated from ADSCs, HFB and adipose tissue using Nucleospin RNA tissue (Machery Nagel).

Cells and tissues were lysed by incubation in the lysis buffer (RA1). After addition of 70% ethanol, the lysate was loaded onto the Nucleospin column and centrifuged at 11,000g for 1 min, to permit RNA to bind at the membrane. Contaminating DNA was removed using rDNase solution, which was directly applied onto the silica membrane.

Samples were washed with two different buffers to remove salts and metabolites. Pure RNA was finally eluted with RNase-free water and quantified at NanoDrop (Thermo Scientific).

**cDNA synthesis**

Total RNA (500 ng) was mixed with 50 ng of random nonamers primer, dNTP mix (10 mM each) and RNase-free water, in a final volume of 12 µl. The reaction was heated to 65°C for 5 min and quick-chilled on ice. Then the First-Strand Buffer (Invitrogen) and 0.1 M DTT were added 5×. After incubation at 25°C for 2 min, 1 µl (200 units) of SuperScript™ II enzyme (Invitrogen) was added.
The reaction was incubated at 42°C for 50 min and inactivated by heating at 70°C for 15 min.

**Real-time PCR**

cDNA was amplified by real-time PCR using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the specific assay designed for the investigated genes. SYBR® Green assays reactions were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA). Each reaction contained 10 µl 2× Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA), 400 nM concentration of each primer and cDNA.

Forward and reverse primers for the selected genes were designed using primer express software (Applied Biosystems, Foster City, CA), and are listed in Table 1.

All experiments were performed including non-template controls to exclude reagent contamination. PCRs were performed with two biological replicates.

Expression was quantified using qRT-PCR. The gene expression levels were normalized to the expression of the housekeeping gene RPL13. Quantification was done with the delta/ delta calculation method13.

**Statistical analyses**

Comparison of gene expression between three types of cells was performed with two-tailed ANOVA using Excel spreadsheets (Microsoft Office 2003).

### Table 1  Primer sequences for SYBR® green assay

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences (5´–3´)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td>F-CTTCGGACCCAATAACAGT R-AGTGTGCTCCGCTGATG</td>
</tr>
<tr>
<td>BDFN</td>
<td>F-CGTGAGGTTTGTGAGCC T-CCACCTTGTCTCGGATGTT</td>
</tr>
<tr>
<td>RPL13</td>
<td>F-CTGGAGAGAAGAGGAAAGA R-TTGAGACCTCTTGATGTCAA</td>
</tr>
</tbody>
</table>

**Figure 2:** Relative gene expression in ADSCs compared with HFb.

**Results**

ADSCs were phenotypically characterized by flow cytometric analyses. Cell preparations derived from adipose tissue were homogenously CD105+, CD90+, CD34−, CD45−, CD14−, which is a typical MSC surface antigen profile (Figure 1).

The expression level of two growth factors, NGF and BDFN, was compared in three cell types: adipocytes, HFb and ADSCs.

Gene expression was quantified using qRT-PCR and normalized to the expression of the housekeeping gene RPL13.

Comparing expression levels of ADSCs and HFb, we found that NGF gene was over-expressed in stem cells. Conversely BDFN was down-regulated (Figure 2).

These two genes were down-regulated in adipocyte when compared with fibroblast (Figure 3) and stem cells.

Stem cells show up-regulation of BDFN and NGF with respect to adipocyte (Figure 4).

**Discussion**

Recent studies have shown that adipose tissue is a paracrine and endocrine organ, able to produce signal proteins termed adipokines, proteins involved in the regulation of energy balance, insulin sensitivity and glucose tolerance, vascular homeostasis, lipid metabolism, angiogenesis and inflammation6,14.

Adipokines are responsible for the complex processes of adipose tissue remodelling with repair of peripheral nerves and changes in the density of blood vessels.

Adipose tissue is innervated by sympathetic nerves and secretes two neurotransmitters, NGF and BDFN, involved in the stimulation of lipolysis15; control of cell number and growth and maintenance of sympathetic neurons within tissues16.

Their expression could influence the good outcome of structural fat grafting, a procedure largely employed in craniomaxillofacial...
In this study, we have compared the ability of ADSCs and adipocytes, derived from the same patient, to secrete these two neurotrophic factors. The expression levels of NGF and BDNF were measured using qRT-PCR.

The expression level of NGF and BDNF was compared in three cell types: adipocytes, stem cells isolated from adipose tissue and dermal fibroblasts.

Obtained results showed that ADSCs over-expressed NGF and BDNF genes, compared with adipocyte, indicating that stem cells are the main producers of neurotrophic factor in adipose tissue.

NGF expression is also up-regulated in stem cells in respect to dermal fibroblast.

Adipose tissue is typically used in structural grafting to restore the dermal tissue of origin. Evidence that stem cells from adipose tissue over-expressing NGF in respect to fibroblasts make these cells a useful source for tissue regeneration.

Conclusion
These results demonstrate that the production of NGF and BDNF is due to the stem cells present in the adipose tissue rather than adipocytes.

The frequency of stem cells within adipose tissue ranges from 1:100 to 1:1500 cells, which far exceeds the frequency of marrow stromal cells (MSCs) in the bone marrow.

Adipose tissue is an ideal source of autologous stem cells, particularly in comparison with the traditional bone marrow cells procurement procedure, as it is easily obtainable by liposuction under local anaesthesia with minimal discomfort to the patient, and its MSC content is adequate for clinical-grade cell manipulation in regenerative medicine.

References

Licensee OA Publishing London 2013. Creative Commons Attribution License (CC-BY)