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Short Communication

Neurogenic differentiation of human adipose-derived stem cells: Relevance of different signaling molecules, transcription factors, and key marker genes

Alejandra Johana Cardozo [a], Daniel Eduardo Gómez [b,1], Pablo Francisco Argibay [a,*]

[a] Instituto de Ciencias Básicas y Medicina Experimental, Hospital Italiano de Buenos Aires, Potosí 4240, Ciudad Autónoma de Buenos Aires (1199), Argentina
[b] Laboratorio de Oncología Molecular, Universidad Nacional de Quilmes, R. S. Peña 352, Bernal (1876), Buenos Aires, Argentina

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ABSTRACT

Since numerous diseases affect the central nervous system and it has limited self-repair capability, a great interest in using stem cells as an alternative cell source is generated. Previous reports have shown the differentiation of adipose-derived stem cells in neuron-like cells and it has also been proved that the expression pattern of patterning, proneural, and neural factors, such as Pax6, Mash1, Ngn2, NeuroD1, Tbr2 and Tbr1, regulates and defines adult neurogenesis. Regarding this, we hypothesize that a functional parallelism between adult neurogenesis and neuronal differentiation of human adipose-derived stem cells exists. In this study we differentiate human adipose-derived stem cells into neuron-like cells and analyze the expression pattern of different patterning, proneural, neural and neurotransmitter genes, before and after neuronal differentiation. The neuron-like cells expressed neuronal markers, patterning and proneural factors characteristics of intermediate stages of neuronal differentiation. Thus we demonstrated that it is possible to differentiate adipose-derived stem cells in vitro into immature neuron-like cells and that this process is regulated in a similar way to adult neurogenesis. This may contribute to elucidate molecular mechanisms involved in neuronal differentiation of adult human non-neural cells, in aid of the development of potential therapeutic tools for diseases of the nervous system.

1. Introduction

Since numerous diseases affect the central nervous system, identifying regulators that control stem cell self-renewal and neuronal differentiation is essential for the development of stem cell-based cell replacement therapies. It is known that the neurodegenerative diseases and brain injuries lead to neuronal loss and that the neural tissue has long been regarded as restricted in regeneration capacity (Reynolds and Weiss, 1992; Richards et al., 1992). But stem cells, with ability to self-renew and differentiate into multiple lineages, are found in different tissues and adipose tissue is an abundant source of adipose-derived stem cells (ASCs) (Gronthos et al., 2001; Hauner et al., 1987; Zuk et al., 2001). Human ASCs have capacity to differentiate in vitro into neuron-like cells (Anghileri et al., 2008; Ashjian et al., 2003; Cardozo et al., 2010; Dhar et al., 2007; Jang et al., 2010; Safford et al., 2002), and in vivo may contribute to functional benefits in a wide range of neurological insults (Chi et al., 2010; Kang et al., 2003; Kim et al., 2007; Kulikov et al., 2008; Wei et al., 2009).

One of the most important issues in stem cell biology is to uncover molecular mechanisms underlying stem cell self-renewal and differentiation. The transition from an undifferentiated to a fully differentiated neural cell comprises a series of sequential steps: proliferation, commitment, specification, and terminal differentiation (Temple, 2001). Throughout neuronal differentiation process different transcription factor families are expressed, such as proneural genes, patterning factors, neuronal and neurotransmitter genes.

Proneural genes are a set of transcriptional regulators that code for basic Helix–Loop–Helix (bHLH) proteins, which play a central role in the differentiation of neural progenitors into neurons and influence the particular neuronal subtypes, produced in a region-specific manner. The expression of proneural bHLH proteins is both necessary and sufficient to promote the generation of differentiated neurons from undifferentiated progenitor cells (Bertrand et al., 2002; Brunet and

Abbreviations: ASCs, adipose-derived stem cells; bHLH, basic Helix–Loop–Helix; Mash, mammalian achaete-scute homolog 1; Math, mammalian atonal homolog 1; Ngn, neurogenin; NeuroD, neurogenic differentiation; Tbr, T-box brain; Pax6, paired box gene 6; BHA, butylated hydroxyanisole; RA, retinoic acid; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; CP, crossing point; DCX, doublecortin; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; NSE, neuron specific enolase; NF200, neurofilament 200 kDa; M1, cholinergic muscarinic receptor 1; GABA, gamma-aminobutyric acid; GABRA1, GABA receptor type A subunit α1; GABAbR1, GABA receptor type B subunit 1; GABAbR2, GABA receptor type B subunit 2; GABRD, GABA receptor type A subunit δ; NKCC1, Na^+, K^+, $2Cl^-$ co-transporter 1; KCC2, K^+/Cl^- co-transporter 2; GluK5, glutamatergic receptors type kainate subtype 5; mGluR5, metabotropic glutamate receptor 5; NMDA, N-methyl-D-aspartic acid; NR1, NMDA 1 receptor.

* Corresponding author at: Instituto de Ciencias Básicas y Medicina Experimental, Hospital Italiano de Buenos Aires, Potosí 4240 – 8° piso – C1199ACL, Ciudad Autónoma de Buenos Aires, Argentina. Tel.: +54 11 4959 0200x8919; fax: +54 11 4959 0200x4155.
E-mail addresses: degomez@unq.edu.ar (D.E. Gómez), pablo.argibay@hospitalitaliano.org.ar (P.F. Argibay).
[1] Tel./fax: +54 11 4365 7257.

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Ghysen, 1999; Nieto et al., 2001; Ross et al., 2003; Sun et al., 2001; Tomita et al., 2000). Two classes of proneural genes can be distinguished: the determination factors, such as mammalian achaete-scute homolog 1 (Mash1), mammalian atonal homolog 1 (Math1), and neurogenin (Ngn), expressed early in mitotic neural precursor cells; and the differentiation factors, including neurogenic differentiation 1 (NeuroD1), NeuroD2, T-box brain 1 (Tbr1), Tbr2 and Math2, expressed later in post-mitotic cells (Bertrand et al., 2002; Morrison, 2001). These proneural genes are downstream effectors of paired box gene 6 (Pax6), a transcription factor that promotes neurogenesis and it is involved in the establishment of the progenitors that produce different classes of neurons followed by oligodendrocytes and astrocytes (Hack et al., 2005; Heins et al., 2002; Sugimori et al., 2007).

Cooperation among patterning, proneural and inhibitory HLH proteins establishes a molecular code that determines both the spatial and the temporal patterns of neurogenesis and gliogenesis, by establishing distinct profiles of proneural gene expression in different progenitor domains and by modulating the neurogenic or gliogenic activity of proneural proteins (Scardigli et al., 2001; Sugimori et al., 2007).

Although the molecular control of neuronal differentiation of hASCs is unknown, we suggest that it would exist a parallelism with the differentiation of adult generated neural cell-types, and that many of the regulatory control genes expressed during adult neurogenesis are also present in our neuronal differentiation protocol, providing an in vitro model of how generation of neuronal-like cells from adult stem cells might occur.

The aim of the present work is to study the expression of different patterning factors, proneural, neuronal, and neurotransmitter genes, before and after the neuronal differentiation of hASCs.

2. Materials and methods

2.1. Cell isolation and culture

After informed consent and approval of the ethics committee of research protocols from the Hospital Italiano de Buenos Aires, adipose tissue samples were obtained during abdominal and mammary plastic surgeries of 23 healthy donors between 26 and 56 years old. The adipose tissue was extensively washed with Hank's balanced salt solution (HBSS, Sigma, Buenos Aires, Argentina) to remove blood and, fibrous material and vessels were carefully dissected and discarded. The remaining tissue was finely minced and digested with 0.1% of Collagenase Type I (Gibco, California, USA) for 45 min with gentle agitation. Enzyme activity was neutralized with a twofold volume of standard medium containing Dulbecco's modified Eagle medium (DMEM, Gibco) with 20% of fetal bovine serum (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (Gibco), and centrifuged for 12 min at 400×*g*. The supernatant containing the lipid droplets was discarded. The stromal vascular fraction settled at the bottom was resuspended in standard medium and seeded in culture dishes (Nunc International, Roskilde, Denmark). Stromal vascular fraction cultures were incubated at 37 °C in a 5% CO2 atmosphere. After 48 h, no adherent cells were removed. When they reached 70–80% of confluence, adherent cells were trypsinized (0.25% at 37 °C for 5 min, Sigma), harvested, and washed with standard medium to remove trypsin and then expanded in larger dishes. A homogenous cell population of hASCs was obtained after 2 or 3 weeks of culture. Cells at early passages (3–5) in culture were used for the experiments.

2.2. Neuronal differentiation

Neuronal differentiation of 12 samples was initiated at passages 3–5 using a modification of previous neuronal induction protocols (Levy et al., 2003; Mareschi et al., 2006; Tao et al., 2005; Woodbury et al., 2000; Zuk et al., 2002). Briefly the cells were plated in dishes until they were subconfluent. Preinduction was performed for 48 h after discarding the medium, washing the cells, and adding new DMEM containing 20% fetal bovine serum and 1 mM β-mercaptoethanol (Riedel, De Haën, Germany). Then, the preinduction medium was removed and the induction medium was added to the culture. The composition of the induction medium was: DMEM with 100 μM butylated hydroxyanisole (BHA, Sigma), 10–6 M retinoic acid (RA, Sigma), 10 ng/ml epidermal growth factor (EGF, Invitrogen, Brazil), and 10 ng/ml basic fibroblast growth factor (bFGF, Invitrogen). Cells were incubated in this medium during 14 days. The medium was changed every 3 days. The cells were monitored continually after neuronal induction and were lysed for ribonucleic acid (RNA) or protein extraction or fixed for immunostaining. One non-induced culture dish was also analyzed in every experiment as control.

2.3. RNA isolation and reverse transcription

Total RNA from hASCs before and after 14 days of neuronal induction was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's recommendations. The purity and integrity of the extracted RNA were evaluated by optical density measurements (260:280 nm ratios) and by visual observation of samples on agarose (Biodynamics, Buenos Aires, Argentina) gels.

Two micrograms of each total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) to eliminate possible contamination of genomic deoxyribonucleic acid (DNA), no amplification was detected in a PCR using treated RNA as sample. One microgram of treated RNA was used as template in a 20 μl volume cDNA synthesis reaction using ImProm-II™ Reverse Transcriptase (Promega).

2.4. Quantitative real-time PCR

Quantification was performed using real-time polymerase chain reaction (real-time PCR) to determine the relative expression levels of proneural, neuronal and neurotransmitter genes involved in neural commitment and terminal differentiation. For this purpose between 6 and 12 samples of induced and non-induced hASCs were analyzed. Real-time PCR reactions were optimized to proceed without the formation of primer dimmers or ectopic bands, which would interfere with quantification. For quantitative real-time PCRs, SYBR Green (Invitrogen), Platinum Taq Polymerase (Invitrogen), and LightCycler 2.0 Instrument (Roche Applied Science) were used. After 45 PCR cycles, a melting curve of the PCR product was obtained. A smooth sigmoid was an indication that the only doubled-strand DNA present in the PCR product was the dimerized product, and no primer dimmers or ectopic bands were contaminating the reaction. To verify the identity of amplified cDNAs, the size of the PCR products was checked on agarose gel.

A calibration standard curve was created for each primer set by serial dilution of a cDNA pool of all samples analyzed. The number of cycles after which the fluorescence of a reaction rose above baseline was designated as crossing point (CP). As more cDNA was included in a reaction, the CP dropped, and a calibration curve of volume of cDNA against CP was plotted. The CP of each cDNA sample was then plotted onto this calibration curve, thus allowing relative cDNA quantification across PCR reactions.

Primer sequences were designed using LightCycler Probe Design Software 2.0 (Roche Applied Science, Mannheim, Germany) using gene sequences obtained from the GeneBank database (Table 1).

The expression of human β-actin gene was used to standardize gene expression levels. The experiments were done in quadruplicates. Control experiments without cDNA template revealed no non-specific amplification. When PCR results were negative, cDNAs from human cell lines or tissues were run as positive controls in order to eliminate the possibility of false negative results.