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Human mesenchymal stromal cell therapy for damaged cochlea repair in *nod-scid* mice deafened with kanamycin

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Abstract

Background. Kanamycin, mainly used in the treatment of drug-resistant-tuberculosis, is known to cause irreversible hearing loss. Using the xeno-transplant model, we compared both *in vitro* and *in vivo* characteristics of human mesenchymal stromal cells (MSCs) derived from adult tissues, bone marrow (BM-MSCs) and adipose tissue (ADSCs). These tissues were selected for their availability, *in vitro* multipotency and regenerative potential *in vivo* in kanamycin-deafened *nod-scid* mice. *Methods.* MSCs were isolated from informed donors and expanded *ex vivo*. We evaluated their proliferation capacity *in vitro* using the hexosaminidase assay, the phenotypic profile using flow-cytometry of a panel of surface antigens, the osteogenic potential using alkaline phosphatase activity and the adipogenic potential using oil-red-O staining. MSCs were intravenously injected in deafened mice and cochleae, liver, spleen and kidney were sampled 7 and 30 days after transplantation. The dissected organs were analyzed using lectin histochemistry, immunohistochemistry, polymerase chain reaction (PCR) and dual color fluorescence *in situ* hybridization (DC-FISH). *Results.* MSCs showed similar *in vitro* characteristics, but ADSCs appeared to be more efficient after prolonged expansion. Both cell types engrafted in the cochlea of damaged mice, inducing regeneration of the damaged sensory structures. Several hybrid cells were detected in engrafted tissues. *Discussion.* BM-MSCs and ADSCs showed *in vitro* characteristics suitable for tissue regeneration and fused with resident cells in engrafted tissues. The data suggest that paracrine effect is the prevalent mechanism inducing tissue recovery. Overall, BM-MSCs and ADSCs appear to be valuable tools in regenerative medicine for hearing loss recovery.

Key Words: *adipose-derived stem cells, bone marrow mesenchymal stromal cells, cochlea, hybrid cells, kanamycin, multipotency, paracrine effect, proliferative capacity, tissue regeneration, xenotransplantation*

Introduction

Deafness is a sensory disability that affects millions of people worldwide. In mammals, deafness is frequently a consequence of various traumas, ototoxic drugs, loud noise or ageing. Deafness in adults is permanent because hair cells and auditory neurons of the organ of Corti (OC) cannot be regenerated after birth. However, recent improvements in stem cell technology have increased studies aimed to prevent or replace lost sensorineural cells, developing regenerative therapies for hearing loss [1–5].

The replacement of sensorineural cells with an exogenous cell source has been recently obtained [2] but further investigations are required [6]. Preliminary studies on cochlear inner ear cell regeneration have been performed *in vivo* by direct injection of embryonic [7,8], neural [9] or mesenchymal stromal cells (MSCs) [10,11] in cochlear compartments. However, the methods of stem cell administration directly into the cochlea are invasive and unsuitable for clinical applications.

Pluripotent endogenous stem cells were found in the vestibular organ of adult mice, although limited

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to the utricular sensorial epithelium [12]. Among early studies on inner ear cell regeneration, bone marrow and hematopoietic stem cells (HSCs) have been shown to engraft in the inner ear and produce MSCs and fibrocytes [13]. Transplantation of MSCs in the cochlea of a rat model with acute sensorineural hearing loss due to fibrocyte dysfunction favored hearing recovery through regeneration of cochlear fibrocytes [14]. Bone marrow-derived HSCs showed the ability to engraft in the cochlea of mice deafened after ototoxic treatment, but they were not able to spontaneously transdifferentiate into any cochlear cell type and preserved their hematopoietic identity [15]. Recently, CD133+ stem cells isolated from human umbilical cord blood and intravenously (IV) injected into irradiated adult *nod-scid* mice, deafened by treatment with kanamycin (an ototoxic aminoglycoside) and/or intense acoustic noise, caused a significant cochlear repair, inducing regeneration of endogenous sensory hair cells and neurons [2].

Other studies indicated that the heterogeneous cell population of mesenchymal stroma contained pluripotent MSCs, among which were bone marrow stem cells and adipose-derived stem cells [16]. Transplanted MSC or undifferentiated mesenchymal progenitor cells have been shown able to *in vivo* engraft in areas of recent physical traumas or chemically induced injuries [17].

The potential of MSCs as repair system for tissues and cells [18] suggested an extension of investigations on the therapeutic ability of MSCs for inner ear repair after acoustic injury. We tested the regenerative ability of human adult mesenchymal cells isolated from bone marrow (BM-MSCs) and adipose-derived stem cells (ADSCs) on a xenotransplanted mouse model, verifying their pluripotent ability *in vitro* and their ability *in vivo* to home into the cochlea of acoustically deafened mice and recover the induced tissue damage.

Materials and methods

Human MSCs

Isolation and expansion ex vivo

BM-MSCs were isolated from bone marrow aspirates of three adult donors, who gave their informed consent. The nucleated cell fraction from each BM-MSC sample was obtained using an automated procedure using a SEPAX S-100 machine (Biosafe) as previously described [19]. Briefly, total nucleated cells (TNCs) were seeded in 75 cm^3 flasks (1.6×10^5/cm^2) in complete growth medium (CGM), Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (Gibco BRL), supplemented with 2 mmol/L L-glutamine, 20% fetal calf serum (FCS; EuroClone) and 1% antibiotics in solution (penicillin 100 U/mL, streptomycin 0.25 mg/mL). Flasks were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 (Passage P 0). The MSC expansion was carried out according to published methods [20,21]. Briefly, expansion of the cells was obtained with successive cycles of trypsinization and reseeding; the expansion was maintained until P12.

Human ADSCs were isolated from subcutaneous (SC) adipose tissue of eight female donors (aged 28–42 years) who gave their informed consent. Fresh lipoaspirates were washed in phosphate-buffered saline (PBS), pH 7.3, then minced in DMEM (Gibco BRL) supplemented with 2 mmol/L L-glutamine, penicillin (50 μg/mL) and streptomycin (50 μg/mL) and digested adding collagenase (1.5 mg/mL, Sigma) at 37°C for 30 min. Fragments were centrifuged (300 *g* for 3 min), washed in the same medium containing 10% FCS, further minced, incubated at 37°C and filtered through a 250 μm nylon mesh. Erythrocytes were removed using erythrocyte lysis buffer (Sigma) and the centrifugation was repeated three times to enrich the ADSC-abundant pellet; approximately 1×10^6 TNCs were usually generated from 2 mL of lipoaspirate. At P0, adherent cells developed into visible colonies 2–3 days after the initial plating. After 5–7 days in culture, the medium was changed for fresh culture medium fetal calf serum (CM-FCS); the cells were then maintained under conditions identical to those used for BM-MSCs.

When required, cryopreservation was performed after P2: human MSCs from both tissue sources were washed and the pellet suspended in cryopreservation medium containing 20% FCS and 10% dimethyl-sulfoxide (DMSO; Sigma). Aliquots were stored at −80°C for the first 24 h and then in liquid nitrogen.

Proliferation assay

The BM-MSCs and ADSCs (4 U each) were cultured in CGM up to 60 days. Cells at P2 were used for experiments. Conventional hexosaminidase colorimetric assay was performed to evaluate cell proliferation [20]. Briefly, cells were washed with PBS and soaked in 200 μL hexosaminidase substrate, 3.75 mmol/L p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma), 0.25% Triton-X 100 (Sigma) and 0.05 mol/L citrate buffer, pH 5.0 (Sigma), and seeded in a 48-well plate. After 1 h of incubation in a humidified atmosphere containing 5% CO_2 at 37°C, the reaction was terminated by adding 75 μL of 5 mmol/L ethylenediaminetetraacetic acid (EDTA) and 50 mmol/L glycine, pH 10.4 (Sigma) in each well. The optical density of the supernatant was read in a 96-well plate reader Sirio (SeacSrl),at 405 nm.

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