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Regeneration of mandibular defects using adipose tissue mesenchymal stromal cells in combination with human serum-derived scaffolds

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ABSTRACT

Bone regeneration is a challenging issue. Traditional solutions bring risks, potential complications, and morbidity. The aim of the present study was to regenerate critical-sized mandible defects in athymic rats with adipose tissue mesenchymal stromal cells (AT-MSCs) in combination with human serum-derived scaffolds. Two approaches to treatment were performed. The first approach used differentiated stromal cells that became osteogenic cell lines. The second approach used no pre-differentiation. Follow-up periods were 45 days and 90 days. Both cell types were combined with human serum-derived scaffolds. Afterward, histological (haematoxylin–eosin and Masson's Trichrome stain modified by Goldner), immunohistochemical (human vimentin and Stro-1), and radiological (microCT) studies were performed. The level of calcification between the groups was compared by analysis of variance, and statistical significance was set at $p < 0.05$. The results demonstrate that bone regeneration can be achieved with both undifferentiated and pre-differentiated cells, but that the structure and level of calcification were better achieved with pre-differentiated cells ($p < 0.05$). The scaffold is suitable for this cell type, is osteoconductive and simple to perform. This article highlights the possible application of adipose tissue mesenchymal stromal cells in combination with a non-mineralized scaffold in bone regeneration.

1. Introduction

Bone regeneration in cranio-maxillofacial surgery is a challenging issue. Traditional solutions include autologous bone grafts in possible combination with bone-derived or synthetic substitutes (Drosse et al., 2008). Although good results can be achieved with these approaches, they carry risks, potential complications and morbidity.

To overcome these problems, tissue engineering using stem cells could both obtain autologous bone substitutes without great morbidity to patients and become a valid and cost-effective alternative. In these respects, there are good clinical results with the use of mesenchymal stromal cells (MSCs) (Wolff et al., 2013; Sándor et al., 2014).

MSCs were originally described as mononuclear cells originating from bone marrow (BM) that are able to adhere to plastic culture dishes and acquire morphology similar to fibroblasts in *in vitro* culture (Friedenstein et al., 1970). These cells differ from haematopoietic stromal cells and exhibit multipotency, meaning

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that they are able to differentiate into mesodermal lineage cells such as bone, cartilage, and adipose cells (Caplan, 1991). MSCs from BM (BM-MSCs) aspirates have been the most studied and most widely used cells with which to perform bone tissue engineering (Caplan, 2007). Furthermore, adipose tissue is an important source of MSCs (AT-MSCs), is easy to obtain, and is an abundant cell source, offering less morbidity to patients and being less cost-effective due to the short time required to amass enough cells for bone regeneration (Gronthos et al., 2001; Zuk et al., 2001; Scherberich et al., 2010; Han et al., 2014).

Different biomaterials can serve as scaffold to regenerate bone. An ideal bone scaffold must be osteoconductive, which lets the bone cells adhere, proliferate, and form extracellular matrix on its surface and pores. The scaffold should also be able to induce new bone formation through biomolecular signalling and recruiting of progenitor cells, together a property known as osteoinduction. Furthermore, an ideal scaffold needs to form blood vessels in or around the implant within a few weeks of implantation to actively support nutrient, oxygen, and waste transport (Olszta et al., 2007). Granular forms present more surface area for cellularization and incorporate more quickly into host tissues than block forms, which remodel more slowly (Leong et al., 2003). The problem with granules is their tendency to migrate, and thus they must be retained at the site of intended implantation by a containment mesh. Therefore, an ideal scaffold must retain both advantages from blocks (management and load-bearing ability) and granules. Multi-scale porous scaffolds involving both micro- and macro-porosities can perform better than purely macro-porous scaffolds (Woodard et al., 2007).

Basically, there are three ways to regenerate bone using AT-MSCs. One involves pre-differentiation into progenitor osteogenic cells (Jurgens et al., 2011), the second is to combine those cells with ossification factors (Jeon et al., 2008; Wolff et al., 2013; Sándor et al., 2014), and the third approach is to use mineralized scaffolds or bioactive glasses with AT-MSCs without any pre-differentiation (Hattori et al., 2006; Wolff et al., 2013; Sándor et al., 2014), with the mineralization of the scaffold being the critical factor for success. Recently, success was achieved with non-mineralized scaffolds that combined AT-MSCs without pre-differentiation with silk fibroin (Correia et al., 2014) and titanium granules (Dahl et al., 2013).

In the present study, two approaches of treatment using AT-MSCs are presented, and their capabilities of regenerating bone *in vivo* using as a model a critical size mandibular defect in athymic rats (Arosarena et al., 2003; Schliephake et al., 2008, 2009) is evaluated. These AT-MSCs were combined with a novel non-mineralized multi-scale pored scaffold, which consists of human serum-derived proteins cross-linked with glutaraldehyde, representing a novel combination in bone regeneration.

2. Material and methods

2.1. AT-MSCs cultures

A total of 1 cm^2 of subcutaneous adipose tissue was collected from the thighs of patients who underwent microsurgical reconstruction. Informed consent was obtained prior to surgery. All experimental protocols were approved by the Institutional Ethics Committee of the Hospital Central Universitario de Asturias (08/04/2011, Code: 28/2011).

The samples were processed as follows to obtain AT-MSCs: a sample was washed three times in phosphate-buffered saline solution (PBS; PAA Laboratories GmbH, Cölbe, Germany), cut, and placed in a test tube. After adding 0.1% collagenase I (Sigma–Aldrich, Madrid, Spain) in Dulbeccos's modified Eagle's medium (DMEM, Gibco, Invitrogen, Paisley, UK), the suspension was shaken and digested for 1 h at 37 °C. Digestion was stopped by adding foetal bovine serum (FBS; Gibco, Invitrogen) to DMEM. After filtering through a 0.40-μm cell strainer (BD Bioscience, Madrid, Spain) and centrifuging at 500 *g* for 10 min, the cells were seeded onto 25-cm^2 polystyrene flasks (Cultek, Madrid, Spain) in an AT-MSCs culture medium consisting of low-dose glucose DMEM (Gibco, Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin (Gibco, Invitrogen) and 100 μg/mL streptomycin (Gibco, Invitrogen). Cells were cultured in a controlled atmosphere (37 °C, 5% CO_2) over 48 h. The medium was first replaced, and the unattached cells were removed. Thereafter, the medium was replenished every 2–3 days, and between the 7th and 8th days, when confluent, the cells were digested with trypsin (0.25% w/v; Gibco, Invitrogen) and plated onto two 75-cm^2 flasks (Cultek, Madrid, Spain). The same procedure was followed again, and cells were used after confluence (passage 2–3).

2.2. AT-MSCs molecular characterization by flow cytometry

The antibodies used against the surface marker set to identify MSCs were those proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (Dominici et al., 2006). The antibodies were against CD34-PerCpCy5.5 (BD Bioscience), CD73-Pe (BD Bioscience), CD90-FITC (eBioscience, Barcelona, Spain), CD105-APC (eBioscience), and CD45-PeCy7 (eBioscience). The cells used were from passage 2–3. Upon confluence, cells from one 75-cm^2 flask were removed by trypsin-EDTA (Lonza, Barcelona, Spain) and washed with 1× PBS. The cells were then incubated with FcR-blocking reagent (eBioscience) for 20 min at 4 °C to avoid non-specific antibody binding. Next, cells were stained with the aforementioned antibodies and their isotype controls for 30 min at 4 °C. Fluorescence minus one controls were stained in parallel using the panel of antibodies with sequential omission of one antibody. After washing, the cells were resuspended in 0.5 mL 1 × PBS and sieved using a 35-μm nylon filter cell strainer (Becton Dickinson, Madrid, Spain). Samples were analysed using a Becton Dickinson FACSAria with FACSDiva 6.1.2 software (Becton Dickinson).

2.3. Scaffold construction

The scaffold was elaborated as previously described according to patent WO2008/119855 (Meana et al., 2008). Briefly, a 10-mL quantity of human venous blood was obtained from a blood bank and kept at 37 °C for 30 min. Next, the blood was centrifuged for 15 min at 2,000 *g*, and the resultant serum (5 mL) was cross-linked with 0.5 mL of 25% glutaraldehyde (Merck, Darmstadt, Germany) and transferred to a 5-mL disposable syringe. After the serum/glutaraldehyde solution was kept at room temperature for 30 min until solidification, it was frozen and kept at −80 °C overnight. Next, the syringe was cut open, and the frozen solution was lyophilized for 48 h and rehydrated in a graded ethanol series (100%–90%–80%) with 1-hour immersions in each dilution. The tube-shaped scaffold was then cut into 4-mm-diameter, 1- to -2-mm thick sections and sterilized in ethanol (70%) for 8 h. Finally, the scaffolds were neutralized in DMEM. Before cell seeding, excess fluid was removed, and the scaffolds were placed in a 24-well culture plate (Nunc, Darmstadt, Germany) with one scaffold per well.

2.4. Cell seeding into scaffolds

Confluent cultures of AT-MSCs were trypsinized and counted in a Neubauer haemocytometer. The cells released were suspended in DMEM containing 10% FBS. Next, 1 × 10^6 cells (250 mL) were