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Bone regeneration in critical-size calvarial defects using human dental pulp cells in an extracellular matrix-based scaffold

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ABSTRACT

The rat calvarial defect is an established model to evaluate craniofacial bone regeneration using cell-scaffold biocomplexes. Dental pulp harbors stem cells with significant osteogenic properties. Extracellular matrix (ECM)-like scaffolds simulate the environment that cells observe *in vivo*. In the present study, we evaluated the osteogenic effect of a biocomplex of human dental pulp cells and a hyaluronic-based hydrogel scaffold in calvarial defects of immunocompetent rats. Dental pulp cells at the 2nd passage were characterized by flow cytometry, osteodifferentiated *ex vivo* for 4 days and the whole population was encapsulated in the synthetic ECM matrix. Cell vitality was verified 24 h upon encapsulation. 5 mm calvarial defects were created in 30 male rats and filled with the biocomplex, the scaffold alone, or left untreated. Histological evaluation at 8 weeks showed incomplete bone regeneration in all groups. The scaffold was not fully degraded and entrapped cells were detected in it. Histomorphometry showed statistically significant superior new bone formation in the biocomplex-treated group, compared to the two other groups. The present study provides evidence that the whole population of human dental pulp cells can advance bone healing when transplanted in immunocompetent animals and highlights the importance of proper scaffold degradation in cell-driven bioengineering treatments.

1. Introduction

Bone tissue regeneration is one of the most challenging aspects of tissue engineering with a great impact on the lives of millions of people worldwide (Greenwald et al., 2001). Although autogenous bone grafts constitute the 'gold standard' of bone defect reconstruction, their inherent donor-related limitations and resorption issues in large defects render their clinical use difficult and sometimes ineffective (Dinopoulos et al., 2012). Bone tissue engineering emerges as the treatment of choice in bone reconstitution by exploiting different combinations of the basic bioengineering tools: stem cells, scaffolds and growth factors (Langer and Vacanti, 1993).

The use of progenitor/stem cells in the engineering of osseous defects is associated with limitations such as locating easily accessible donor sites and isolating cell populations with adequate self-renewal capacity and specific differentiation potential; human dental pulp stem cells (DPSCs) seem to circumvent these problems. These ectomesenchymal-derived cells exhibit notable trans-differentiation capability *in vitro* depending on the culture conditions (Gronthos et al., 2002; Kawashima, 2012) and significant osteogenic potential in particular (Laino et al., 2005; Riccio et al., 2010). Recent findings support the potential use of human dental pulp stem cells in the treatment of osseous defects. In rodents, subcutaneous implantation of scaffolds seeded with human DPSCs resulted in ectopic bone formation (Laino et al., 2005; d'Aquino et al., 2007; Yang et al., 2009; Chan et al., 2011), while their orthotopic use in calvarial bone defects has led to significant healing (Riccio et al., 2012; Prisciotta et al., 2012; Maraldi et al., 2013).

A prerequisite for a successful bone engineering approach is the use of biomaterials that support the optimum function of the

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osteogenic progenitor/stem cells. The hybrid synthetic hydrogels possess the biological characteristics of the inherent extracellular matrix (ECM), thus providing the incorporated cells with the necessary environmental cues, whereas the simultaneous addition of synthetic polymers allows for the optimal modification of their mechanical properties (Salinas and Anseth, 2009). A certain type of hydrogel scaffolds, called synthetic extracellular matrix (sECM), consists of chemically modified hyaluronic acid, gelatin and a polymer solution that allows encapsulation of stem cells and the construction of three-dimensional cell cultures. It thus creates an environment that better mimics the *in vivo* conditions (Shu et al., 2006). Despite these remarkable properties, the performance of these scaffolds has not yet been evaluated adequately in bone tissue engineering and this was the rationale of using a material of this type in the present study.

Until now, the main achievement in the field of bone repair, by using cells of dental origin, has been the combination of osteoinductive scaffolds with a specific subpopulation of DPSCs that has shown exceptional results in calvarial defects of immunosuppressed rats (Riccio et al., 2012; Pisciotta et al., 2012; Maraldi et al., 2013). In our opinion, it would be very interesting to investigate the effectiveness of the totality of dental pulp cells, encapsulated in a sECM scaffold, on the healing of calvaria defects in rodents. A favorable outcome of this combination would render the application of bone engineering more feasible in clinical practice, by diminishing at a great extent the relative efforts and cost. Since the early inflammatory response contributes to the initial process of tissue repair and given the immunosuppressive properties of DPSCs (Pierdomenico et al., 2005; Wada et al., 2009) immunocompetent rodents could also be used in the above model.

The research hypothesis of the present study was that the treatment of calvarial bone defects in non-immunosuppressed rats with a biocomplex of human dental pulp cells encapsulated within a hyaluronic-based hydrogel scaffold could lead to enhanced bone regeneration, compared to defects receiving either no treatment or treatment with non-cell seeded scaffolds. The specific aims were to characterize the mesenchymal-like potential of the used human dental pulp population and to evaluate the *in vivo* scaffold suitability and osteogenic effect of the transplanted biocomplexes by using histology and histomorphometry.

2. Materials and methods

The experimental protocol followed in this study has been approved by the Ethics Committee of the School of Dentistry, National and Kapodistrian University of Athens and all animal treatments were performed according to the guidelines of the European Communities Council Directive of 22 September 2010 (2010/63/EU) on the ethical use of animals.

2.1. Isolation, culture and characterization of human dental pulp cells

2.1.1. Primary culture and osteoinduction of pulp cells

The pulp was collected from normal immature impacted third molars extracted for orthodontic reasons in the School of Dentistry, National and Kapodistrian University of Athens, after patients' informed consent, in compliance with the Greek legislation. Immediately after extraction, the teeth were swabbed with sterile gauze, cut with a diamond bur in two pieces and then transferred into a culture hood where the pulps were removed with forceps and minced into tiny pieces with scalpels under sterile conditions. The explants were cultured at 37 °C, 5% CO_2 and 95% humidity in DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, Life Technologies Co., Carlsbad, CA, USA), 100 UI/ml penicillin, 100 μg/ml streptomycin (Lonza), and 0.25 μg/ml amphotericin B (Fungizone®, Gibco, Invitrogen), as previously described (Diamanti et al., 2013). The culture medium was changed every other day. Confluent cultures were collected by trypsinization (trypsin/EDTA, Lonza) and subcultured. Cells at the 2nd passage were cultured in the presence of osteoinductive factors [50 μg/ml ascorbic acid, 10^{-8} M dexamethasone, 10 mM β-glycerophosphate (Sigma Aldrich, St. Louis, MO, USA)] for 4 days and were subsequently used for the preparation of cell-scaffold biocomplexes.

2.1.2. Flow cytometry

Dental pulp cells before and after osteoinduction were analyzed by flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA) for the detection of surface stem cell markers. The cells (1×10^6) were incubated for 30 min at 4 °C in the dark with the following mouse monoclonal anti-human antibodies: fluorescein isothiocyanate (FITC)-conjugated CD44, FITC-CD90, phycoerythrin (PE)-conjugated CD166 and PE-CD34 (BD Biosciences). The antigens' choice was based on previous literature referring to relevant surface markers of dental pulp stem cells (Kawashima et al., 2012). Following incubation, the cells were washed twice with phosphate buffered saline (PBS, without Ca^{2+} and Mg^{2+}). The supernatant was removed by centrifugation for 5 min at 1500 $\times g$ and the cell pellet was resuspended in PBS and analyzed in FACSCalibur. These measures were repeated in three different occasions.

2.2. Preparation and analysis of biocomplexes

2.2.1. Preparation of scaffolds and cell encapsulation

Three-dimensional constructs were prepared by incorporating pulp cells within a hydrogel scaffold (HyStem™-HP Cell Scaffold Kit, Sigma Aldrich), composed by hyaluronic acid, heparin sulphate, gelatin and PEDGA solution. Initially, the scaffold components were mixed according to the manufacturer and placed in molds (5 mm diameter, 1 mm thickness), prepared in the interior of 1 ml sterile insulin syringes. Subsequently, the osteoinduced cells were encapsulated (10^6/scaffold) into the scaffold templates by gently mixing with a pipette. Following full polymerization of the material, the resulting biocomplexes were liberated from the syringes by moving the piston and placed into 12-well plates with osteoinductive medium for 1 more day. Scaffolds without cells were also prepared with the same procedure.

2.2.2. Cell viability and growth within the biocomplexes

Cell encapsulation and viability within the scaffold were microscopically evaluated 1 day after the biocomplex preparation. Specifically, the biocomplexes were fixed with 4% neutral formalin solution, washed with blocking solution (BSA 3% and Tween 0.5% in PBS) and incubated with 4,6-diamino-2-phenylindone (DAPI-Invitrogen, 1/2000 in PBS) for 20 min in order to stain the nuclei of viable cells. For the observation under a fluorescence microscope (Leica Microsystems, Wetzlar, Germany), the biocomplexes were placed on slides and pressed with a coverslip. Formalin-fixed biocomplexes were also embedded in paraffin. Microtome sections (6 μm) were used to detect the cell proliferation marker Ki67 by immunohistochemistry using a primary antibody for human Ki67 (clone MIB1, Dako Corporation, Glostrup, Denmark). Slides were observed and photographed under an optical microscope (DMLB2, Leica).

2.3. Animals and surgical procedures

In total, thirty male Wistar rats 4 months old at the time of surgery were used for the *in vivo* experiments. Animals were