Acta Biomaterialia xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Direct comparison of current cell-based and cell-free approaches towards the repair of craniofacial bone defects – A preclinical study

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ARTICLE INFO

Article history:
Received 1 April 2015
Received in revised form 9 July 2015
Accepted 13 August 2015
Available online xxxx

Keywords:
Bone tissue engineering
Bone graft
Calcium phosphate
Mesenchymal stromal cells
Bone marrow
Calvaria

ABSTRACT

For craniofacial bone defect repair, several alternatives to bone graft (BG) exist, including the combination of biphasic calcium phosphate (BCP) biomaterials with total bone marrow (TBM) and bone marrow-derived mesenchymal stromal cells (MSCs), or the use of growth factors like recombinant human bone morphogenic protein-2 (RhBMP-2) and various scaffolds. Therefore, clinicians might be unsure as to which approach will offer their patients the most benefit. Here, we aimed to compare different clinically relevant bone tissue engineering methods in an "all-in-one" study in rat calvarial defects. TBM, and MSCs committed or not, and cultured in two- or three-dimensions were mixed with BCP and implanted in bilateral parietal bone defects in rats. RhBMP-2 and BG were used as positive controls. After 7 weeks, significant *de novo* bone formation was observed in rhBMP-2 and BG groups, and in a lesser amount, when BCP biomaterials were mixed with TBM or committed MSCs cultured in three-dimensions. Due to the efficacy and safety of the TBM/BCP combination approach, we recommend this one-step procedure for further clinical investigation.

Statement of Significance

For craniofacial repair, total bone marrow (BM) and BM mesenchymal stem cell (MSC)-based regenerative medicine have shown to be promising in alternative to bone grafting (BG). Therefore, clinicians might be unsure as to which approach will offer the most benefit. Here, BM and MSCs committed or not were mixed with calcium phosphate ceramics (CaP) and implanted in bone defects in rats. RhBMP-2 and BG were used as positive controls. After 7 weeks, significant bone formation was observed in rhBMP-2 and BG groups, and when CaP were mixed with BM or committed MSCs. Since the BM-based procedure does not require bone harvest or cell culture, but provides *de novo* bone formation, we recommend consideration of this strategy for craniofacial applications.

1. Introduction

Patients with craniofacial defects often require bone repair. Autologous bone grafting (BG) is considered to be the gold standard method [77]. However, this technique displays several limitations, which are mostly related to the harvesting of bone at a

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http://dx.doi.org/10.1016/j.actbio.2015.08.013
1742-7061/

secondary operative site [13,69]. The use of bone substitutes especially biphasic calcium phosphate (BCP) allows for the avoidance of secondary site morbidity and reduces surgery time compared to BG [16,64]. However, although BCP contributes to bone healing through osteoconduction, it generally lacks osteoinductivity for regenerating large defects or in tissues exposed to sources of contamination. Therefore, clinical applications must be restricted to small defects or to regions with significant bone interaction [78]. In light of these limitations, surgeons and researchers have worked to develop alternative therapies to BG over the past fifteen years. These new approaches have mainly involved combining osteoprogenitor cells or growth factors with bone substitutes in order to enhance their osteoinductive properties. In fact, extemporaneous mixtures of BCP and unprocessed total bone marrow (TBM) have shown osteogenic potential *in vivo*. This technique induced bone formation in extraosseous sites [62,63] and potentiated bone ingrowth at osseous sites [6,34,35,40,41,42,59,63]. However, it seems that the main limitation to TBM-based strategies is donor-to-donor variability, which cannot be predicted *a priori*. These differences between individuals are likely related to the variable amounts of mesenchymal stromal cells (MSC) contained within distinct bone marrow samples. As a result, it can be difficult to obtain reproducible levels of bone formation in clinical settings.

Since Friedenstein et al. demonstrated the osteogenic potential of TBM-derived MSC [31,32,33], the combination of these cells with various scaffolds has emerged as a treatment strategy. Indeed, these bone tissue engineering (BTE) techniques have led to promising results in both small animal models [12] and clinically-sized implants (orthotopic and ectopic) [45,65]. Nevertheless, the efficacy of BTE in humans remains to be clinically validated [15,56,67]. Notably, recent developments in BTE technology, including three-dimensional (3D) cell culture in bioreactors [71,76] and gene therapy, have improved the ability of engineered bone to act as a substitute for autologous BG. Despite a large body of evidence highlighting the preclinical potential of these advanced therapies, studies examining "head to head" comparisons of these various strategies are still lacking. In addition, investigations into the most efficient method for bone regeneration are fundamentally important for helping clinicians to offer their patients the safest and most efficacious treatments. Since the clinical use of these advanced therapies remain in its infancy, learning more about each of the distinct bone repair strategies is necessary to address any uncertainties surrounding their potential therapeutic relevance.

The aim of the present study was to compare the bone formation potential of nine well-known repair strategies involving BCP granules, MSC or growth factors. Moreover, we assessed if (multi-step) BTE techniques were more efficient than so called "*in vivo* BTE procedures", such as TBM combined with BCP or recombinant human bone morphogenic protein-2 (rhBMP-2)-soaked collagen sponges (single step).

2. Materials and methods

All procedures involving animals were conducted in accordance with the institutional guidelines of the French Ethical Committee (CEEA.PdL.06), and efforts were made to minimize suffering.

2.1. Materials

Cell culture materials were purchased from Corning (Schipol-Rijk, the Netherlands). Sodium L-ascorbate, vitamin D3, dexamethasone, Alizarin Red S, insulin-transferrin-selenium media supplement, 3-isobutyl-1-methylxanthine, indomethacin, Oil Red O, and trypan blue were purchased from Sigma–Aldrich (St. Louis, MO). Alpha minimum essential medium (αMEM), phosphate-buffered saline (PBS), penicillin–streptomycin, trypsin–EDTA (0.05%–0.53 mM), L-glutamine, TRIzol® reagent, and Superscript III Kits were obtained from Invitrogen (Paisley, UK). Fetal calf serum (FCS) was provided by Dominique Dutscher (Brumath, France). Beta-glycerophosphate was purchased from Calbiochem (Darmstadt, Germany). Brilliant SYBR® Green Master Mix was obtained from Stratagene (Amsterdam Zuidoost, the Netherlands). Polymerase chain reaction (PCR) primers were synthesized by MWG Biotech (Ebersberg, Germany), and Turbo DNase was purchased from Ambion Inc. (Applied Biosystems; Courtaboeuf, France). All other chemicals were obtained from standard laboratory suppliers and were of the highest purity available.

2.2. Calcium phosphate biomaterials

BCP particles (MBCP™; 500–1000 μm) were made of hydroxyapatite (60%) and beta-tricalcium phosphate (40%) (Biomatlante; Vigneux de Bretagne, France). Tubes containing granules (0.015 g each) were double-packed and autoclave sterilized at 121 °C for 20 min.

2.3. Animals

Thirty-three adult inbred Lewis 1A-haploype RT1a rats were obtained from a certified breeding center (Charles River, l'Arbresle, France) and acclimatized for 2 weeks to the conditions of the local vivarium.

2.4. Bone marrow harvesting

Three rats were specifically designated as TBM, BG, and MSC donors. Animals were anesthetized using inhaled isoflurane (Forene; Abott, Rungis, France) and sacrificed via intracardiac overdose of sodium thiopental (Nesdonal; Rhône-Merieux, Lyon, France). Rat TBM was isolated from femurs and tibias for extemporaneous grafting and MSC isolation. Briefly, the ends of each bone were cut, and 1 mL of TBM mixed with saline was obtained through an intramedullary bone flush procedure performed with a 26-gauge needle. After pooling, the TBM was immediately transferred to heparinized tubes (Venoject I; Terumo Europe, Louvain, Belgium). Cytology and myelography were performed as previously described [28].

2.5. Cancellous bone harvesting

Cancellous bone was harvested using a curette from the epiphyses of bones previously cut for TBM harvesting. The bone was crushed then implanted into cranial defects.

2.6. Isolation and expansion of bone marrow-derived mesenchymal stromal cells

A portion of the total harvested TBM volume was filtered through a 70-μm nylon mesh filter. The TBM was seeded in tissue culture treated polystyrene flasks, and MSC were isolated based on their adherence capacity after 2 days as described previously [22]. Cells were then cultured in proliferative medium (PM), consisting of αMEM supplemented with 1% L-glutamine, 1% penicillin/streptomycin, and 10% FCS. They were subsequently incubated at 37 °C in a humidified atmosphere (5% CO_2). The medium was renewed twice a week until the cells were 80–90% confluent. The cells were then enzymatically detached from the flasks through incubation with 0.25% trypsin/EDTA (3–4 min) and counted using a Malassez hemocytometer and trypan blue exclusion dye. To obtain a large amount of cells, MSC were further expanded in treated polystyrene

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