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Porous decellularized tissue engineered hypertrophic cartilage as a scaffold for large bone defect healing

Gráinne M. Cunniffe [a,b,c], Tatiana Vinardell [d], J. Mary Murphy [e], Emmet M. Thompson [a,f], Amos Matsiko [a,f], Fergal J. O'Brien [a,b,c,f], Daniel J. Kelly [a,b,c,f,*]

[a] Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland
[b] Department of Mechanical and Manufacturing Engineering, School of Engineering, Trinity College Dublin, Dublin 2, Ireland
[c] Advanced Materials and Bioengineering Research Centre, Trinity College Dublin & RCSI, Dublin 2, Ireland
[d] School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland
[e] REMEDI, National Centre for Biomedical Engineering Science, National University of Ireland Galway, Galway, Ireland
[f] Tissue Engineering Research Group, Dept. of Anatomy, Royal College of Surgeons in Ireland, Dublin 2, Ireland

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ABSTRACT

Clinical translation of tissue engineered therapeutics is hampered by the significant logistical and regulatory challenges associated with such products, prompting increased interest in the use of decellularized extracellular matrix (ECM) to enhance endogenous regeneration. Most bones develop and heal by endochondral ossification, the replacement of a hypertrophic cartilaginous intermediary with bone. The hypothesis of this study is that a porous scaffold derived from decellularized tissue engineered hypertrophic cartilage will retain the necessary signals to instruct host cells to accelerate endogenous bone regeneration. Cartilage tissue (CT) and hypertrophic cartilage tissue (HT) were engineered using human bone marrow derived mesenchymal stem cells, decellularized and the remaining ECM was freeze-dried to generate porous scaffolds. When implanted subcutaneously in nude mice, only the decellularized HT-derived scaffolds were found to induce vascularization and *de novo* mineral accumulation. Furthermore, when implanted into critically-sized femoral defects, full bridging was observed in half of the defects treated with HT scaffolds, while no evidence of such bridging was found in empty controls. Host cells which had migrated throughout the scaffold were capable of producing new bone tissue, in contrast to fibrous tissue formation within empty controls. These results demonstrate the capacity of decellularized engineered tissues as 'off-the-shelf' implants to promote tissue regeneration.

1. Introduction

The regeneration of large bone defects through tissue engineering approaches has typically attempted to induce direct osteogenic healing by recapitulating the process of intramembranous ossification. A potential drawback with this approach is that mineralized tissue initially deposited around the periphery of the engineered graft can prevent host vascularization of the implant, leading to the establishment of a necrotic core [1]. In the developing embryo however, most bones form *via* the process of endochondral ossification, the progressive replacement of a hypertrophic cartilaginous template with bone. During endochondral ossification MSCs condense, differentiate down the chondrogenic lineage and become hypertrophic [2]. These hypertrophic chondrocytes secrete biomolecules such as vascular endothelial growth factor (VEGF), which are known to promote angiogenesis, and thus actively induce ingrowth of vasculature as the surrounding matrix is calcifying, in addition to promoting matrix remodelling [3–5]. Hence, it has been suggested that strategies attempting to recapitulate the process of endochondral ossification may provide a superior approach for healing large bone defects [6–10].

A number of studies have demonstrated the capacity of *in vitro* tissue engineered cartilage to undergo endochondral ossification and generate bone tissue *in vivo* [11–14]. For example, cartilaginous tissues engineered using human bone marrow derived mesenchymal stem cells (MSCs) have been shown to act as a template for host cell-derived bone formation following subcutaneous implantation into nude mice [15]. Tissue engineering such an autologous cartilaginous graft for clinical applications would involve isolating and expanding the patient's cells, forming a

* Corresponding author at: Department of Mechanical and Manufacturing Engineering, School of Engineering, Trinity College Dublin, Dublin 2, Ireland. Tel.: +353 18963947.
E-mail address: kellyd9@tcd.ie (D.J. Kelly).

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construct of suitable dimensions to fit the original defect, culturing this construct for a number of additional weeks *in vitro* and finally implanting it into the body. There are a number of challenges that would need to be overcome before clinical translation of such a cell-based therapy, including the high cost, complexity, need for two surgical procedures and associated regulatory hurdles [16]. Additionally, with large bone defect healing, increasing graft size can become a major issue, with new bone formation occurring primarily around the periphery of the implant, leading to the search for a suitable osteoinductive material to amplify the ossification process throughout the regenerating tissue. Furthermore, 'off-the-shelf' availability may well determine the extent to which any new therapy achieves widespread clinical adaption. This has led to increased interest in the use of decellularized extracellular matrix (ECM) derived scaffolds for the regeneration of different tissue types [17–22], based on the premise that such biomaterials contain structural and functional molecules to facilitate tissue regeneration. The ECM can be harvested from native tissue or from cell-derived matrix deposited during *in vitro* culture [23–28]. In the context of endochondral bone regeneration, it remains unclear as to whether decellularized hypertrophic cartilage would retain its pro-angiogenic ability and thus its capacity to direct host-mediated orthotopic bone regeneration.

The hypothesis of this study is that a porous scaffold derived from decellularized hypertrophic cartilage engineered using allogeneic bone marrow derived MSCs will be highly osteoinductive, support vascularization, and will promote bone formation by host progenitor cells. To test this hypothesis, porous scaffolds derived from either engineered cartilage tissues (CT) or engineered hypertrophic cartilage tissues (HT) were implanted subcutaneously into nude mice to evaluate their potential to promote vascularization and ectopic bone formation. The capacity of these HT scaffolds to promote bone regeneration within critically-sized rat femoral defects was then evaluated. The data presented in this study suggest that engineered HT derived scaffolds could be used to improve the repair of critically-sized bone defects.

2. Experimental section

2.1. Experimental design

This study consisted of two parts. The first experiment initially established the feasibility of generating porous scaffolds entirely from cell-derived ECM, and then compared the ability of scaffolds generated from either engineered cartilage tissue (CT) or engineered hypertrophic cartilage tissue (HT) to promote vascularization and induce ectopic bone formation in a subcutaneous nude mouse model. CT and HT was engineered through self-assembly of human bone marrow-derived MSCs (see specific details below), from which acellular CT and HT scaffolds were generated and implanted into nude mice. Viable tissue engineered grafts were also implanted as positive controls. The second experiment then evaluated the optimum scaffold type from the subcutaneous study to promote bone regeneration in a critically-sized rat femoral defect. Again, this scaffold was implanted acellularly and the study investigated the ability of host cells to infiltrate the structure and induce bone repair.

2.2. Generating scaffolds for subcutaneous implantation

Adult human bone marrow-derived stem cells (hMSCs) were expanded to passage 2 and seeded into 6.5 mm diameter transwell inserts (3.0 μm pore polycarbonate membrane, Corning) at a density of $4 \times 10^{\wedge 6}$ cells per insert [29,30]. Constructs were cultured in either chondrogenic medium (consisting of hgDMEM GlutaMAX supplemented with 100 U/mL penicillin/streptomycin (both Gibco), 100 μg/mL sodium pyruvate, 40 μg/mL L-proline, 50 μg/mL L-ascorbic acid-2-phosphate, 4.7 μg/mL linoleic acid, 1.5 mg/mL bovine serum albumin, 1× insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma–Aldrich), 2.5 μg/mL amphotericin B, and 10 ng/mL of human transforming growth factor-β3 (TGF-β3; Prospec-Tany TechnoGene Ltd)) for 6 weeks at 5% O_2 or cultured in chondrogenic medium for the first 4 weeks and switched to hypertrophic medium (consisting of hgDMEM GlutaMAX supplemented with 100 U/mL penicillin/streptomycin, 100 μg/mL sodium pyruvate, 40 μg/mL L-proline, 50 μg/mL L-ascorbic acid-2-phosphate, 4.7 μg/mL linoleic acid, 1.5 mg/mL bovine serum albumin, 1× insulin–transferrin–selenium, 1 nM dexamethasone, 2.5 μg/mL amphotericin B, 1 nM L-thyroxine, and 10 mM β-Glycerophosphate (both Sigma–Aldrich)) at 20% O_2 for the final 2 weeks to induce deposition of a hypertrophic cartilage ECM (Fig. 1A). Both cartilage tissue (CT) and hypertrophic cartilage tissue (HT) constructs were then collected directly after *in vitro* culture and homogenized (IKAT10, IKA Works Inc., NC, USA) to form separate CT and HT slurries (500 mg/ml) in distilled H_2O, placed in custom designed polydimethylsiloxane (PDMS) cylindrical moulds (wells measuring 5 mm diameter, 3 mm height) and freeze-dried to create porous 5 mm × 3 mm constructs (Fig. 2A). Lyophilization was carried out in a freeze-dryer (Triad, Labconco) by bringing the samples to a final freezing temperature of −30 °C at a ramp speed of 1 °C min^{-1}, with a holding step at −30 °C for 1 h to allow for ice-crystal nucleation and growth [25]. The samples were then brought to a drying temperature of −10 °C at the same ramp speed and maintained under vacuum (0.1 mbarr) for 24 h to ensure full drying was achieved. No chemical decellularization techniques were applied to these constructs prior to lyophilization, as these scaffolds were implanted into immunocompromised mice. All scaffolds were cross-linked and sterilized using dehydrothermal (DHT; 24 h at 105 °C) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC; 6 mM for 2 h with two washes in sterile phosphate buffered saline 2 × 1 h) treatments prior to implantation [31]. The hMSCs in this study were used with approval from the National University of Ireland, Galway and University College Hospital ethics committees and after informed consent.

2.3. Generating scaffolds for orthotopic rat femoral defect study

Only HT constructs were used to generate scaffolds for this defect study, based on results from the initial nude mouse study. HT grafts were cultured *in vitro* as before, using hMSCs in a scaffold-free system to deposit hypertrophic cartilage matrix. The resulting blended HT slurry was then decellularized using detergent (0.1% SDS, 1 h) and enzymatic (10 U/ml DNase, 10 U/ml RNase, 1 h) treatments with washes, before freeze-drying in custom designed polydimethylsiloxane (PDMS) cylindrical moulds (wells measuring 4 mm diameter, 5 mm height) to yield porous decellularized scaffolds (4 mm × 5 mm) for implantation into a rat femoral defect. This decellularization protocol was included in order to remove DNA content while retaining ECM components, and hence reduce the risk of eliciting an immune reaction following implantation. Constructs were freeze-dried, cross-linked and sterilized as described in Section 2.2.

2.4. Scanning Electron Microscopy (SEM)

Scaffolds were imaged using SEM to visualize the internal porous structure of the freeze-dried constructs. Imaging was carried out following DHT treatment by fixing constructs to an adhesive carbon stub, sputter coating with gold, and imaging using a lens

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