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Engineering cartilage or endochondral bone: A comparison of different naturally derived hydrogels

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ABSTRACT

Cartilaginous tissues engineered using mesenchymal stem cells (MSCs) have been shown to generate bone in vivo by executing an endochondral programme. This may hinder the use of MSCs for articular cartilage regeneration, but opens the possibility of using engineered cartilaginous tissues for large bone defect repair. Hydrogels may be an attractive tool in the scaling-up of such tissue engineered grafts for endochondral bone regeneration. In this study, we compared the capacity of different naturally derived hydrogels (alginate, chitosan and fibrin) to support chondrogenesis and hypertrophy of MSCs in vitro and endochondral ossification in vivo. In vitro, alginate and chitosan constructs accumulated the highest levels of sulfated glycosaminoglycan (sGAG), with chitosan constructs synthesizing the highest levels of collagen. Alginate and fibrin constructs supported the greatest degree of calcium accumulation, though only fibrin constructs calcified homogeneously. In vivo, chitosan constructs facilitated neither vascularization nor endochondral ossification, and also retained the greatest amount of sGAG, suggesting it to be a more suitable material for the engineering of articular cartilage. Both alginate and fibrin constructs facilitated vascularization and endochondral bone formation as well as the development of a bone marrow environment. Alginate constructs accumulated significantly more mineral and supported greater bone formation in central regions of the engineered tissue. In conclusion, this study demonstrates the capacity of chitosan hydrogels to promote and better maintain a chondrogenic phenotype in MSCs and highlights the potential of utilizing alginate hydrogels for MSC-based endochondral bone tissue engineering applications.

1. Introduction

Tissue engineering involves using a combination of cells, three-dimensional scaffolds and signalling molecules to repair or regenerate damaged or diseased tissues [1,2]. The use of a supporting scaffold or hydrogel facilitates the scaling-up of tissue engineered grafts to clinically relevant sizes. Furthermore, understanding how cell–scaffold interactions regulate the terminal phenotype of the cell is critical in the development novel tissue regeneration strategies. To date, cell-based bone tissue engineering applications have generally focused on the direct osteogenic priming of mesenchymal stem cell (MSC)-seeded scaffolds in a process resembling intramembranous ossification [3]. This approach, however, has been hampered by insufficient vascularization of the graft following in vivo implantation, thus preventing the necessary delivery of oxygen and nutrients required to ensure cell survival [4]. For example, in vitro osteogenic priming of engineered constructs has been shown to occlude the pores of a scaffold with calcified matrix, resulting in the development of a necrotic core upon implantation into bony defects [5]. Core necrosis is a well-documented challenge in the field of tissue engineering, and will be exacerbated by the scaling-up of such constructs to treat critically sized bone defects.

In an attempt to address these challenges, there has been a recent shift away from classical tissue engineering paradigms, towards strategies aimed at recapitulating the natural mechanisms that drive tissue development during skeletogenesis [6]. The long bones of the body form by a process termed endochondral ossification, whereby chondrocytes in a developing cartilaginous rudiment undergo hypertrophy and direct vascularization and remodelling of the cartilaginous template into bone [7]. An endochondral

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approach to bone tissue engineering may circumvent many of the issues associated with the intramembranous method, as cells progressing down the endochondral route are equipped to survive hypoxic conditions and release pro-angiogenic factors for the conversion of avascular tissue to vascularized tissue [8]. Indeed, it has been demonstrated that MSC-based cartilaginous grafts can be used to generate bone in vivo in both ectopic and orthotopic sites by executing such an endochondral programme [8–15].

Hydrogels are water-swollen cross-linked polymers capable of forming large, abstract shaped constructs [16]. They can be derived from natural materials which are either components of, or have macromolecular properties similar to, native extracellular matrix [17], and a number of naturally derived hydrogels have been shown to support chondrogenesis of MSCs in vitro [18–22]. Previous studies have compared the chondrogenic capabilities of MSC-seeded hydrogels in vitro [23–26], and also the potential of chondrogenically primed MSC-seeded hydrogels to maintain a stable chondrogenic phenotype in vivo [27–29]. However, little is known about the capacity of different MSC-seeded hydrogels to support the development of either phenotypically stable cartilage or endochondral bone in vitro and in vivo.

Therefore, the objective of this study was compare the capacity of different naturally derived hydrogels (alginate, chitosan and fibrin) to support chondrogenesis and hypertrophy of MSCs in vitro, and subsequent endochondral ossification in vivo. In the first phase of the study, MSCs were encapsulated in alginate, chitosan and fibrin hydrogels, and cultured in chondrogenic conditions (5 weeks) in order to promote chondrogenesis and cartilaginous matrix production. Thereafter, constructs were switched to hypertrophic conditions (3 weeks) in order to promote a hypertrophic phenotype and the development of calcified cartilaginous tissue. In the second phase of the study MSCs were encapsulated in alginate, chitosan and fibrin hydrogels and subjected to a shorter in vitro culture period (6 weeks) prior to subcutaneous implantation into nude mice for an additional 6 weeks.

2. Materials and methods

2.1. Isolation and expansion of MSCs

Bone marrow derived MSCs were isolated from the femoral shaft of 4 month old pigs and expanded according to a modified method for human MSCs [30] in high-glucose Dulbecco's modified Eagle's medium GlutaMAX (hgDMEM) supplemented with 10% v/v fetal bovine serum (FBS), 100 U ml^{-1} penicillin/100 μg ml^{-1} streptomycin (all Gibco Biosciences, Dublin, Ireland) and 2.5 μg ml^{-1} amphotericin B (Sigma–Aldrich, Dublin, Ireland) at 20% pO_2. Porcine MSCs were chosen for this study as pigs are similar to humans in terms of genetics, anatomy and physiology [31], and previous work in our laboratory has demonstrated the tri-potentiality of MSCs derived from porcine tissue [32]. Following colony formation, MSCs were trypsinized, counted, seeded at density of 5×10^3 cells cm^{-2} in 500 cm^2 triple flasks (Thermo Fisher Scientific), supplemented with hgDMEM, 10% v/v FBS, 100 U ml^{-1} penicillin/100 μg ml^{-1} streptomycin, 2.5 μg ml^{-1} amphotericin B and 5 ng ml^{-1} human fibroblastic growth factor-2 (FGF-2; Prospec-Tany TechnoGene Ltd., Israel) and expanded to passage 2.

2.2. Encapsulation of MSCs within alginate, chitosan and fibrin hydrogels

A 4% agarose-based mould was used to cast cylindrical (5 mm diameter × 3 mm) alginate, chitosan and fibrin hydrogels at a cell density of 20×10^6 MSCs ml^{-1}. Alginate constructs were fabricated by injecting passage 2 MSC-laden 2% w/v alginate (Pronova, FMC Biopolymer, Norway) into a 4% agarose/50 mM $CaCl_2$ mould, and allowing gelation to occur for 30 min. Chitosan constructs were fabricated by combining 1.5 ml of 3% w/v chitosan (Pronova) with 350 μl of β-glycerophosphate (β-GP; 600 mg ml^{-1}; Sigma–Aldrich, Dublin, Ireland), 360 μl of hydroxyethylcellulose (HEC; 25 mg ml^{-1}; Sigma–Aldrich) and 790 μl^{-1} of MSC suspension and allowing gelation to occur at 37 °C for 30 min to yield a final concentration of 1.5% w/v chitosan, 7% w/v β-GP and 0.3% w/v HEC. Fibrin constructs were fabricated by dissolving 100 mg ml^{-1} bovine fibrinogen (Sigma–Aldrich) in 10,000 KIU ml^{-1} aprotinin (Norma Pharma, UK) containing 19 mg ml^{-1} sodium chloride (NaCl). This solution was laden with MSCs and combined 1:1 with a 5 U ml^{-1} thrombin in 40 mM $CaCl_2$ solution and allowed to gel at 37 °C for 30 min, yielding a final concentration of 50 mg ml^{-1} fibrinogen, 2.5 U ml^{-1} thrombin, 5000 KIU ml^{-1} aprotinin, 17 mg ml^{-1} NaCl and 20 mM $CaCl_2$. The concentrations of fibrinogen and thrombin were selected based on a previous study utilizing these concentrations to form fibrin hydrogels which supported chondrogenic and hypertrophic differentiation of MSCs, and also generated hydrogels of sufficient mechanical strength to be subjected to cyclic dynamic compression within a bioreactor [33].

2.3. In vitro culture conditions

The chondrogenic culture conditions applied in this study are defined as culture in a chondrogenic medium (CM) consisting of hgDMEM GlutaMAX supplemented with 100 U ml^{-1} penicillin/streptomycin (both Gibco), 100 μg ml^{-1} sodium pyruvate, 40 μg ml^{-1} L-proline, 50 μg ml^{-1} L-ascorbic acid-2-phosphate, 4.7 μg ml^{-1} linoleic acid, 1.5 mg ml^{-1} bovine serum albumine, 1 × insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma–Aldrich), 2.5 μg ml^{-1} amphotericin B and 10 ng m^{-1} of human transforming growth factor-β3 (TGF-β3) (Prospec-Tany TechnoGene Ltd., Israel) at 5% pO_2. The hypertrophic culture conditions applied are defined as culture in a hypertrophic medium consisting of hgDMEM GlutaMAX supplemented with 100 U ml^{-1} penicillin/streptomycin, 100 μg ml^{-1} sodium pyruvate, 40 μg ml^{-1} L-proline, 50 μg ml^{-1} L-ascorbic acid-2-phosphate, 4.7 μg ml^{-1} linoleic acid, 1.5 mg ml^{-1} bovine serum albumine, 1 × insulin–transferrin–selenium, 1 nM dexamethasone, 2.5 μg ml^{-1} amphotericin B, 1 nM L-thyroxine (Sigma–Aldrich) and 20 μg ml^{-1} β-GP at 20% pO_2.

2.4. Experimental design

The first phase of this study examined the in vitro development of MSC-seeded alginate, chitosan and fibrin cylindrical hydrogels. Constructs were maintained in chondrogenic culture conditions for a period of 5 weeks, and thereafter were switched to hypertrophic culture conditions for an additional 3 weeks. The second phase of the study investigated the capacity of MSC-seeded alginate, chitosan and fibrin hydrogels to undergo endochondral ossification in vivo. Constructs were maintained in chondrogenic culture conditions for a period of 5 weeks, followed by an additional week in hypertrophic culture conditions. At the end of the 6 week in vitro culture period a single channel (2 mm diameter) was cored into the constructs prior to subcutaneous implantation in nude mice for 6 weeks.

2.5. In vivo subcutaneous implantation

MSC-seeded alginate, chitosan and fibrin hydrogels were implanted subcutaneously into the back of nude mice (Balb/c; Harlan, UK) as previously described [34]. Briefly, two subcutaneous pockets were made along the central line of the spine, one at the shoulders and the other at the hips, and into each pocket three