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Endochondral bone formation in gelatin methacrylamide hydrogel with embedded cartilage-derived matrix particles

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ABSTRACT

The natural process of endochondral bone formation in the growing skeletal system is increasingly inspiring the field of bone tissue engineering. However, in order to create relevant-size bone grafts, a cell carrier is required that ensures a high diffusion rate and facilitates matrix formation, balanced by its degradation. Therefore, we set out to engineer endochondral bone in gelatin methacrylamide (GelMA) hydrogels with embedded multipotent stromal cells (MSCs) and cartilage-derived matrix (CDM) particles. CDM particles were found to stimulate the formation of a cartilage template by MSCs in the GelMA hydrogel *in vitro*. In a subcutaneous rat model, this template was subsequently remodeled into mineralized bone tissue, including bone-marrow cavities. The GelMA was almost fully degraded during this process. There was no significant difference in the degree of calcification in GelMA with or without CDM particles: 42.5 ± 2.5% vs. 39.5 ± 8.3% (mean ± standard deviation), respectively. Interestingly, in an osteochondral setting, the presence of chondrocytes in one half of the constructs fully impeded bone formation in the other half by MSCs. This work offers a new avenue for the engineering of relevant-size bone grafts, by the formation of endochondral bone within a degradable hydrogel.

1. Introduction

Engineering bone through the endochondral pathway has gained increased attention over recent years [1]. This approach is based on our growing knowledge on the formation of long bones in the developing embryo [2] and on the bone fracture healing process [3]. The formation of long bones starts with the condensation of mesenchymal stromal cells (MSCs), which then differentiate into chondrocytes. These chondrocytes secrete a cartilage-specific matrix, rich in collagen type II and glycosaminoglycans (GAGs). In a terminal hypertrophic differentiation process, the chondrocytes subsequently recruit a mixture of cells that is responsible for the ossification and vascularization of the cartilage template [4]. Thus, the hypertrophic chondrocyte is the director of the development of long bones in the human body.

Bone tissue engineering, however, has traditionally focused on mimicking the intramembranous pathway [5,6]. This process is more straightforward than endochondral ossification and naturally occurs in, for example, the formation of the flat bones of the skull [2]. A major drawback to this approach for the repair of large bone defects is the limited size of the engineered tissues [5]. Large engineered bone constructs require rapid vascularization in order to provide cells in the core of the construct with sufficient oxygen and nutrients. A co-culture of osteogenic and vascular progenitor cells or the engineering of advanced prevascularized constructs may offer solutions [5,7,8]. Alternatively, the endochondral approach could overcome this limitation as chondrocytes thrive well in hypoxic conditions [9,10]. Also, the natural healing of bone fractures relies on endochondral ossification within the fracture callus [3]. In this context, chondrogenically pre-differentiated MSC aggregate cultures have shown capable of recapitulating the process of endochondral bone formation *in vivo* [11–13]. Moreover, these hypertrophic cell aggregates and cartilage grafts stimulated endochondral bone formation in segmental bone defects [14,15].

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To increase the size of the endochondral bone forming constructs, a scaffold may be required as a carrier for cells or cell aggregates, as recently shown for biodegradable polymer or organic scaffold materials [13,16,17]. We additionally developed cartilage-derived matrix (CDM), obtained by the decellularization of cartilage tissue [18,19]. Following this process, CDM consists of predominantly collagen type II, in the absence of GAGs and cells. Biochemical cues are supposedly retained in this decellularized biomaterial that can direct the differentiation of cells [18]. We have recently shown that these CDM scaffolds are suitable templates for *in vitro* cartilage matrix formation by seeding MSCs [19]. Nevertheless, hydrogels may be preferred over dry, (in)organic scaffolds, since their degradation profile is tailorable, biochemical cues can easily be incorporated to direct cell fate [20] and they allow for the fabrication of multiphasic constructs [21,22].

Ideally, the hydrogel facilitates the proliferation and condensation of cells, the formation of a cartilage template and subsequent remodeling into bone. Gelatin methacrylamide (GelMA) hydrogel could be a suitable substrate, given the extensive cartilage matrix formation we previously observed in this gel [23,24]. GelMA hydrogels are enzymatically degradable and tunable for specific regenerative applications through modification of the degree of methacrylation and the polymer concentration [24–26]. For example, besides cartilage regeneration, functional vascular networks could be established in GelMA gels [27].

The objective of this study was to engineer relevant-size bone tissue equivalents via the endochondral pathway in a composite of GelMA hydrogel with CDM particles. This composite biomaterial was created in order to benefit from the biological activity of the CDM, while retaining the versatility of the GelMA system. We evaluated *in vitro* cartilage matrix formation by MSCs embedded in GelMA/CDM and subsequent *in vivo* endochondral bone formation in an ectopic rat model. Chondrocytes in GelMA with and without CDM particles served as a control group. In addition, bone formation by MSCs was studied in bilayered constructs with chondrocytes in the cartilage layer to evaluate the potential of GelMA for osteochondral tissue formation.

2. Materials and methods

2.1. Experimental set-up

The objective was to evaluate endochondral bone formation in GelMA hydrogel with embedded equine CDM particles. To determine the optimal concentration of equine CDM particles in the hydrogel, the effect of various concentrations of CDM particles on the GelMA mechanical properties was tested. Chondrogenesis was assessed as the cartilage-specific matrix formation by equine chondrocytes and MSCs in *in vitro* cultures for up to six weeks (Table 1). Both cell types were incorporated in GelMA with and without CDM particles (CDM + and CDM - respectively). After eight weeks of subcutaneous implantation in rats, the same groups were evaluated for chondrogenesis and endochondral bone formation *in vivo*. In addition, bilayered samples were implanted to assess the effect of chondrocytes on the endochondral bone forming capacity by MSCs.

Table 1
Experimental set-up. The chondrogenic differentiation capacity of chondrocytes and MSCs, and the effect of CDM particles in GelMA were tested *in vitro*. Subsequently, chondrogenesis and endochondral bone formation were evaluated subcutaneously in rats. Samples were implanted for eight weeks.

Sample	CDM	*In vitro* 3 donors; replicates (n)	*In vivo* 1 donor; replicates (n)
GelMA	−	−	3
GelMA	+	−	3
GelMA + chondrocytes	−	3	6
GelMA + chondrocytes	+	3	−
GelMA + MSCs	−	3	6
GelMA + MSCs	+	3	6
GelMA *bilayered*: chondrocytes/MSCs	−	−	6

2.2. Production of CDM particles

Full-thickness cartilage was harvested from cadaveric stifle joints (knee) of healthy equine donors (age 3–10 years) that had been euthanized for reasons other than joint disease, with consent of the owners. The cartilage was pooled and decellularized according to a protocol adapted from Benders et al. [19]. In short, after washing the cartilage slices in phosphate-buffered saline (PBS), they were snap-frozen in liquid nitrogen and lyophilized overnight. Then, cartilage was milled in liquid nitrogen (A11 basic analytical mill, IKA, Staufen, Germany) for a few minutes in order to increase exposure to the following chemical treatments, which were all performed on a shaker plate at 2000 rpm. The cartilage particles were treated with six cycles of 0.25% trypsin-EDTA (Invitrogen) at 37 °C for 48 h in total. The particles were then washed in PBS and underwent a 4-hour treatment with a nuclease solution of 50 U/ml deoxyribonuclease (Sigma) and 1 U/ml ribonuclease A (Sigma) in 10 mM Tris–HCl (pH 7.5), at 37 °C. Next, the particles were treated with 10 mM hypotonic Tris–HCl for 20 h followed by 1% (v/v) Triton X-100 in PBS for 24 h, both at room temperature. The particles were thoroughly washed in PBS in 6 cycles for 48 h in total, in order to remove all enzymatic agents. The suspension of cartilage particles in PBS was lyophilized overnight and the pellet was milled again in liquid nitrogen and sieved through pores of 300 μm. The length and width of the CDM particles were measured from 2D light microscope images with ImageJ software (1.46r, National Institutes of Health, USA).

2.3. Isolation of equine chondrocytes and multipotent stromal cells

Macroscopically healthy full-thickness cartilage was harvested under sterile conditions from the stifle joint of fresh equine cadavers ($n = 3$, age 3–10 years) with consent of the owners. After fragmentation and overnight digestion in type II collagenase (Worthington Biochemical Corp) at 37 °C, the suspension was filtered through a 100 μm cell strainer, washed in PBS and stored in liquid nitrogen at −196 °C in culture medium (Dulbecco's Modified Eagle Medium (DMEM) 41965, Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum (FBS, Biowhittaker) and 10% dimethylsulfoxide (DMSO (Merck, Darmstadt, Germany)). Upon thawing, the chondrocytes were seeded at a density of $5 \cdot 10^3$ cells/cm^2 and expanded for 10–12 days in a monolayer culture in chondrocyte expansion medium consisting of DMEM, 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen), and 10 ng/ml FGF-2 (R&D Systems) and embedded in GelMA at passage 1.

With approval of the local animal ethical committee, a bone marrow aspirate from the sternum was obtained from healthy, living equine donors ($n = 3$, age 3–10 years). The mononuclear fraction (MNF) was isolated by centrifuging the sample on Ficoll-Paque (Sigma). The MNF was seeded at a density of $2.5 \cdot 10^5$ cells/cm^2 and expanded in a monolayer culture till sub-confluency in MSC expansion medium containing α-MEM (22561, Invitrogen) complemented with 10% heat-inactivated FBS, 0.2 mM L-ascorbic acid 2-phosphate (Sigma), 100 units/ml penicillin and 100 μg/ml streptomycin, and 1 ng/ml FGF-2 and embedded in GelMA in passages 3–4.

The multilineage potential of the cells that were cultured from the bone marrow aspirate was investigated by a three-way differentiation assay as previously described [19,28,29].

2.4. Preparation of GelMA with CDM particles and cells

GelMA was synthesized by reaction of porcine type A gelatin (Sigma–Aldrich, St. Louis, Missouri, USA) with methacrylic anhydride (Sigma–Aldrich) at 50 °C for one hour, as previously described [24,30]. In short, methacrylic anhydride was added dropwise to a 10% solution of gelatin in PBS under constant stirring. To achieve a high degree of functionalization, 0.6 g of methacrylic anhydride was added per gram of gelatin. The functionalized polymer was dialyzed against distilled water for 7 days at 40 °C to remove methacrylic acid and anhydride, neutralized to pH 7.4 with 10% sodium bicarbonate (Merck, Darmstadt, Germany), freeze-dried and stored at −20 °C until use. This protocol results in a degree of functionalization of circa 75% [24].

Defrosted GelMA was dissolved in PBS at 70 °C at a concentration of 10% (w/v) containing a photoinitiator Irgacure 2959 (Ciba, BASF, Ludwigshafen am Rhein, Germany) at a final concentration of 0.1% (w/v). The CDM particles were mixed (in various concentrations, see Section 2.5) in the GelMA with a positive displacement pipet. Then chondrocytes or MSCs were suspended in the hydrogel at a concentration of $5 \cdot 10^6$ cells/ml for the viability assay and at a concentration of $20 \cdot 10^6$ cells/ml for the *in vitro* and *in vivo* differentiation analyses. For mechanical analyses, cylindrical GelMA samples of 1.3 mm height and 8–9 mm diameter were UV-crosslinked at an intensity of 180 mW/cm^2 (350–450 nm, Hönle UV technology, Munich, Germany). For all other assays, the cell- and CDM-laden GelMA was crosslinked for 15 min in a custom-made air-sealed Teflon mold (sample size ca. 8 × 8 × 2 mm) using 365 nm light in a UVP CL-1000L crosslinker (UVP, Upland, California, USA).

Chondrocyte-laden samples were cultured *in vitro* in chondrogenic differentiation medium (DMEM (41965, Invitrogen) supplemented with 0.2 mM L-ascorbic acid 2-phosphate, 0.5% human serum albumin (SeraCare Life Sciences), 1× ITS-X (Invitrogen), 100 units/ml penicillin and 100 μg/ml streptomycin, 25 mM 4-(2-

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