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Successful chondrogenesis within scaffolds, using magnetic stem cell confinement and bioreactor maturation

Nathalie Luciani [a,*], Vicard Du [a], Florence Gazeau [a], Alain Richert [a], Didier Letourneur [c], Catherine Le Visage [b], Claire Wilhelm [a]

[a] Laboratoire Matière et Systèmes Complexes (MSC), UMR 7057 CNRS & University Paris Diderot, Paris F-75205 Cedex 13, France
[b] INSERM, U791, LIOAD, Nantes F-44042, France
[c] Laboratoire de recherche vasculaire translationnelle, INSERM UMR 1148 & University Paris Diderot, Paris, France

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ABSTRACT

Tissue engineering strategies, such as cellularized scaffolds approaches, have been explored for cartilage replacement. The challenge, however, remains to produce a cartilaginous tissue incorporating functional chondrocytes and being large and thick enough to be compatible with the replacement of articular defects. Here, we achieved unprecedented cartilage tissue production into a porous polysaccharide scaffold by combining of efficient magnetic condensation of mesenchymal stem cells, and dynamic maturation in a bioreactor. In optimal conditions, all the hallmarks of chondrogenesis were enhanced with a 50-fold increase in collagen II expression compared to negative control, an overexpression of aggrecan and collagen XI, and a very low expression of collagen I and RUNX2. Histological staining showed a large number of cellular aggregates, as well as an increased proteoglycan synthesis by chondrocytes. Interestingly, electron microscopy showed larger chondrocytes and a more abundant extracellular matrix. In addition, the periodicity of the neosynthesized collagen fibers matched that of collagen II. These results represent a major step forward in replacement tissue for cartilage defects.

Statement of Significance

A combination of several innovative technologies (magnetic cell seeding, polysaccharide porous scaffolds, and dynamic maturation in bioreactor) enabled unprecedented successful chondrogenesis within scaffolds.

1. Introduction

Osteoarthritis is a chronic progressive musculoskeletal disorder characterized by a gradual loss of hyaline cartilage. It affects a large number of middle-aged people [1], markedly undermining their mobility, quality of life and productivity, and resulting in significant healthcare spending [2–5]. Because of the low turnover of extracellular matrix components in the avascular environment [6], joint cartilage cannot repair itself. Attempts to repair hyaline cartilage have included microfracture, mosaicplasty and autologous chondrocyte implantation (ACI) [7–9], but the long-term efficacy of these approaches is uncertain [10,11]. Research in this setting has also focused on the capacity of mesenchymal stem cells (MSC) to differentiate into chondrocytes [12,13], and to secrete growth factors, cytokines and chemokines [14]. Most techniques currently used to trigger in vitro chondrogenesis of MSC share a cell condensation step, currently achieved through centrifugation or micromass culture, as a prerequisite for commitment to the chondrogenic lineage [15]. We recently produced compact, functional cartilaginous tissues of millimeter dimensions by controlling MSC condensation and fusion with magnets [16]. Besides, aggregates of differentiated MSC incorporated into an in vitro model of cartilage defects showed the same mechanical properties (shear stress) as physiological cartilage [17]. MSC sheet engineering has also been explored as a scaffold-free method of cartilage regeneration [18,19]. Recently, it was shown that MSC can differentiate into three-dimensional pellets in the presence of hyaluronan (HA) microspheres loaded with TGF-β3; HA lubricates the joint and provides mechanical support [20]. A scaffold support thus seems necessary to retain therapeutic cells at the target site and to avoid disruption of the replacement cartilage by frictional shear stress

* Corresponding author.
E-mail address: nathalie.luciani@univ-paris-diderot.fr (N. Luciani).

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[21]. Porous scaffolds can be manufactured from synthetic materials or natural biodegradable polymers [22,23], and can also act as a drug delivery vehicle [24]. Natural polymers have the added advantages of being biocompatible, facilitating cell adhesion, and differentiation [25]. Scaffolds can be designed with a specific 3D shape and stiffness suitable for use in regenerative medicine. Multiphase osteochondral scaffolds based on collagen-glycosaminoglycan and calcium phosphate constructs have already been used to mimic native joint cartilage [26]. However, it has so far proved difficult to induce stem cells to differentiate into chondrocytes and osteocytes when embedded in such scaffolds [27]. One critical parameter is pore size, which must permit both cell condensation and chondrogenic differentiation. The main challenge in seeding a scaffold with cells is to reach a sufficient cell density to achieve a tissue-like cell confinement and organization. To improve cell incorporation, one must use external forces acting on cells at a distance. Magnetic forces are then the most appealing candidates, combining long range action and remote application. Magnetic seeding within scaffold was recently achieved as a valuable strategy to enhance seeding efficacies and to promote cell condensation [28,29].

In this study, we hypothesized that a magnetic seeding and condensation process, that has already been shown to drive chondrogenesis in absence of a scaffold [16], could be used on seeded scaffolds. We selected hydrophilic, non-immunogenic and biodegradable pullulan and dextran polysaccharides [30]. They are both FDA approved for a wide variety of applications in food and pharmaceutical industries. Several groups previously reported pullulan/dextran hydrogels for in vitro culture and differentiation of stem cells [31–33]. Their interconnected porous architecture allows for homogeneous cell distribution and provides a 3D microenvironment with enhanced diffusion of nutrients and oxygen [34]. Here, we demonstrate that pullulan/dextran scaffolds, previously shown to confine stem cells [35], not only retain MSC but also allow them to differentiate into chondrocytes. Using magnetic forces to attract and retain the cells within the scaffold, we enhanced MSC seeding density and condensation. When we combined this magnetic condensation technology with dynamic differentiation in a bioreactor, MSC differentiation into chondrocytes within the scaffold constructs was markedly improved.

2. Materials and methods

2.1. Anionic maghemite nanoparticles

Magnetic nanoparticles were synthesized with the Massart procedure, by alkaline coprecipitation of iron chloride salts [36]. The resulting particles have a maghemite core (γ-Fe_2O_3) 8 nm in diameter and are stabilized in aqueous suspension by adsorption of negatively charged citrate ligands.

2.2. Cell culture, cell labeling and iron quantification

Two different batches of human mesenchymal stem cells (MSC, Lonza, Basel, Switzerland) were cultured in MSCGM medium (Lonza) at 37 °C with 5% CO_2. Magnetic labeling was achieved using iron oxide (maghemite) nanoparticles synthetized by coprecipitation of iron salts, and chelated with citric acid. The stability of the colloidal suspension is thus ensured by electrostatic repulsion thanks to the negative charges brought by the citrate carboxylate groups (COO^-). The nanoparticles exhibit typical superparamagnetic behavior, with no magnetic hysteresis. Their size distribution can be deduced from the fit of their magnetization curve, well described by a log-normal distribution, with mean value of 8.1 nm, and polydispersity of 35%. Size was also measured by TEM, with an average size of 9.4 ± 2 nm, slightly superior than the magnetic size. Prior to incubation with cells, nanoparticles were diluted at a final concentration of [Fe] = 0.2 mM in serum-free RPMI culture medium supplemented with 5 mM free citrate (to avoid precipitation in the culture medium). Cells were then incubated with this solution for 30 min at 37 °C, then rinsed thoroughly in serum-free RPMI medium and incubated overnight with complete MSCGM medium before further processing.

The iron load per cells was quantified by single cell magnetophoresis. Briefly, detached cells were resuspended at a density of 0.2 million per ml, introduced in a 1 mm thick chamber, and submitted to a magnetic force created by a permanent magnet (magnetic field 150 mT, magnetic field gradient 17 mT/mm). The magnetic cell migration towards the magnet is then video-monitored, and the balance of the magnetic force and the viscous drag (provided by the measure of each cell velocity and diameter) leads to the value of each cell magnetic moment, or equivalently mass or iron. More details can be found in [37].

2.3. Porous scaffold preparation and magnetic cell seeding

Polysaccharide scaffolds were prepared from a 75:25 mixture of pullulan/dextran plus the cross-linking agent sodium trimetaphosphate (STMP) at 11% (w/v) in alkaline conditions (10 M NaOH) [38]. Pores were created with a gas-foaming technique using sodium carbonate in 20% acetic acid. Scaffolds were freeze-dried for 48 h to remove water. Rehydration yielded transparent scaffolds with a regular internal lamellar pore structure and a pore size of 185–205 μm.

Scaffolds 7 mm thick with a surface area of 1.8 cm^2 were seeded with 2×10^6 magnetic MSC, using either passive diffusion or magnetic condensation. Three to four scaffolds were seeded for each condition and two to four independent experiments were performed. 1) For magnetic condensation, we used a device composed of 9 small magnets (3 mm in diameter, 6 mm long; total surface area 1.8 cm^2) placed over a permanent neodymium magnet (Fig. 2A). Each magnet created a 150 mT magnetic field and a field gradient of 30 mT/mm in the vicinity of the scaffold. The scaffold was placed in a glass-bottomed cell culture dish (35 mm), and the dish was placed on the magnetic device. Magnetically labeled cells were then gently dropped onto the scaffold. 2) For passive

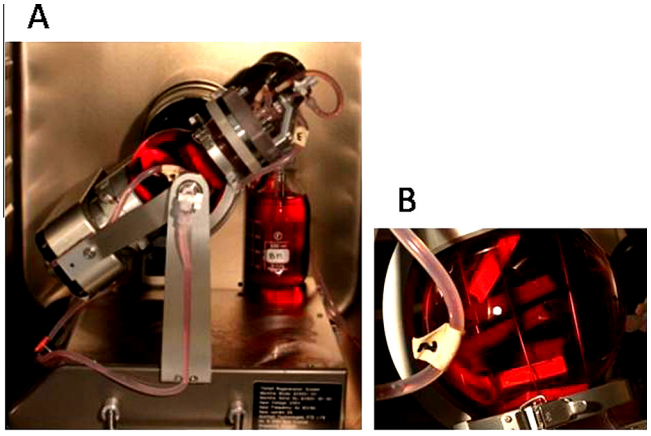

Fig. 1. The TisXell cellular bioreactor (A) applies biaxial rotation, providing continuous irrigation of the 3D scaffold and improving gas exchanges. The arm and chamber rotational speeds can be controlled independently (1–12 rpm and 1–35 rpm respectively). A flow rate of 5 rpm was applied to both the arm and the chamber, as recommended by the constructor for soft 3D tissue regeneration. A peristaltic pump provides a continuous supply of culture medium at 10 rpm. The scaffolds were placed within cages to prevent disruption, and the assemblies were placed in the 500 ml chamber (B) containing differentiation medium.