Acta Biomaterialia 36 (2016) 210–219

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Influencing chondrogenic differentiation of human mesenchymal stromal cells in scaffolds displaying a structural gradient in pore size

Andrea Di Luca [a], Karol Szlazak [b], Ivan Lorenzo-Moldero [a,d], Corina A. Ghebes [c], Antonio Lepedda [e], Wojcech Swieszkowski [b], Clemens Van Blitterswijk [a,d], Lorenzo Moroni [a,d,*]

[a] University of Twente, Tissue Regeneration Department, Drienerlolaan 5, 7522 NB Enschede, The Netherlands
[b] Materials Design Division, Faculty of Material Science and Engineering, Warsaw University of Technology, 141 Woloska Street, 02-507 Warsaw, Poland
[c] University of Twente, Developmental Bio-engineering Department, Drienerlolaan 5, 7522 NB Enschede, The Netherlands
[d] Maastricht University, MERLN Institute for Technology Inspired Regenerative Medicine, Complex Tissue Regeneration Department, P. Debyelaan 25, 6229 HX Maastricht, The Netherlands
[e] Universita'di Sassari, Department of Biomedical Sciences, Via Muroni 25, Sassari, Italy

ARTICLE INFO

Article history:
Received 22 October 2015
Received in revised form 29 February 2016
Accepted 7 March 2016
Available online 8 March 2016

Keywords:
Gradients
3D scaffolds
Additive manufacturing
Stem cells

ABSTRACT

Articular cartilage lesions have a limited ability to heal by themselves. Yet, golden standard treatments for cartilage repair such as drilling, microfracture and mosaicplasty provide further damage and an unstable solution that degenerates into fibrocartilage in time. Articular cartilage presents a number of gradients in cell number and size along with structural gradients in extra cellular matrix (ECM) composition. Therefore, creating scaffolds that display a structural gradient can be an appealing strategy for cartilage tissue regeneration treatments. In the present study, a scaffold with an in-built discrete gradient in pore size was produced by additive manufacturing. Human mesenchymal stromal cells (hMSCs) were seeded within the gradient scaffolds and their proliferation, differentiation and ECM deposition was evaluated with respect to 2 non-gradient scaffolds. Glycosaminoglycan (GAG) deposition was significantly higher in gradient scaffolds and non-gradient scaffolds with the smallest pore size compared to non-gradient scaffolds with the largest pore size. A gradual increase of chondrogenic markers was observed within the gradient structures with decreasing pore size, which was also accompanied by an increasingly compact ECM formation. Therefore, scaffolds displaying a structural gradient in pore size seem to be a promising strategy to aid in the process of hMSC chondrogenic differentiation and could be considered for improved cartilage tissue regeneration applications.

Statement of Significance

We present the development of a novel hierarchical scaffold obtained by additive manufacturing. Structural hierarchy is obtained by changing pore size within the pore network characterizing the fabricated scaffolds and proves to be a functional element in the scaffold to influence adult stem cell differentiation in the chondrogenic lineage. Specifically, in regions of the scaffolds presenting smaller pores an increasing differentiation of stem cells toward the chondrogenic differentiation is displayed. Taking inspiration from the zonal organization of articular cartilage tissue, pore size gradients could, therefore, be considered as a new and important element in designing 3D scaffolds for regenerative medicine applications, in particular for all those tissues where gradient physical properties are present.

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1. Introduction

Cartilage is the tissue located at the extremity of long bones and is responsible for the transmission of forces from the articular surface to the underlying subchondral bone [1]. Cartilage can be affected by progressive degenerative diseases such as osteoarthritis, which involves the osteochondral or chondral tissue depending on the depth of the injury. This process can be triggered by a severe trauma, repetitive minor injuries or aging process [2]. Currently, an optimum treatment for such type of lesions is still under debate. Articular cartilage lesions generally do not heal, or heal only partially under certain biological conditions [3]. Among the available

* Corresponding author at: Maastricht University, Complex Tissue Regeneration Department, Universiteitsingel 40, 6229 ER, The Netherlands.
E-mail address: l.moroni@maastrichtuniversity.nl (L. Moroni).

http://dx.doi.org/10.1016/j.actbio.2016.03.014
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treatments, most of them are based on the disruption of the damaged site such as drilling, microfracture or spongialization [4,5]. Bleeding from the subchondral bone would then ensure the delivery of mononuclear cells and growth factors contained in the bone marrow responsible for the temporary healing of the damaged site. Other proposed techniques consist in the transplantation of autologous cells [6,7], perichondrial/periosteal grafts [8], and autologous or allogenic osteochondral transplantation [9,10]. Since all these proposed solutions present several drawbacks related to morbidity, risks of infections, lack of available tissues, and re-degeneration of the repaired tissue, the use of biomaterial-based strategies to promote and direct tissue growth emerged in the past decades. Several techniques have been proposed in literature to produce scaffolds, such as freeze drying [11], salt leaching [12,13], solvent casting [13] and gas foaming [14]. The success of a scaffold resides in some characteristic features such as total porosity, pore size and interconnectivity, mechanical and physico-chemical properties. The fore mentioned techniques lack control of one or more of these characteristics. Additive manufacturing has been used to fabricate scaffolds due to its ability of overcoming these drawbacks and the possibility to fine tune scaffolds characteristics such as pore shape and size. Additionally, a gradual variation of the scaffolds characteristics can be achieved within the same construct, allowing the design and production of scaffolds displaying structural gradients [15,16].

Gradients are also present in the human body from the developmental phase to adult life. Morphogen gradients are known to lead to the formation of the osteochondral tissue [17]. At the tissue interface, the gradual variation from one tissue to the other is based on gradients. Skin and bone present a gradient structure in the axial direction and the radial direction, respectively. Cartilage is a tissue which displays an internal variation of properties such as stiffness [18–20], extracellular matrix (ECM) composition [2,18] and cell shape and number [21,22] along the axial direction as well. In particular, articular cartilage displays a gradient in proteoglycans, collagen type II, and water content. While proteoglycan content increases from the calcified region to the surface plateau, collagen type II and water content increases from the surface plateau to the calcified zone [23]. Therefore, the use of gradient scaffolds could be a feasible solution for cartilage regeneration. In the present study, scaffolds with a discrete gradient in pore size in the axial direction were fabricated by additive manufacturing. The proliferation, ECM deposition and differentiation of human bone marrow derived mesenchymal stromal cells (hMSCs) toward the chondrogenic lineage were evaluated. Furthermore, we analyzed the differentiation of hMSCs within the compartments of the gradient in order to correlate their differentiation to the size of the pores. We hypothesized that a variation in pore size could indirectly result into a local gradient in nutrient availability during culture. Correspondently, this may result in gradual hypoxic culture conditions, which are known to facilitate chondrogenesis [24,25]. hMSCs have been chosen due to their potential to differentiate into different skeletal lineages, among others [26,27]. Their presence in the mononuclear cell fraction of the subchondral bone marrow further guarantees a potentially straightforward clinical application, as hMSCs are often thought to be responsible for cartilage repair in current clinical surgical procedures such as microfracture.

2. Materials & methods

2.1. Scaffolds preparation

Scaffolds were fabricated via 3D fiber deposition (Bioscaffolder, SysENG, Germany). Scaffolds made of poly(ethylene oxide terephthalate)/poly(butylene terephthalate) (PEOT/PBT) were produced. PEOT/PBT is a family of block co-polymers characterized by an *a*PEOT*b*PBT*c* nomenclature, where *a* is the molecular weight of the starting PEG block and *b* and *c* are the PEOT/PBT ratio. A 300PEOT55PBT45 composition was chosen due to its proven potential for skeletal regeneration [28,29]. Briefly, the polymer (300PEOT55PBT45, PolyVation, The Netherlands) was placed in a stainless syringe and processed at 200 °C. The molten polymer was extruded through a cartridge unit, by the application of a nitrogen flow with a pressure of 5 bar from a pressurized cap and an extrusion screw rotation of 200 rpm.

During plotting, the needle diameter, layer thickness and speed were kept constant at 200 μm, 150 μm and 180 mm/min, respectively. The fiber spacing was kept constant to 500 μm and 1100 μm for non-gradient (NG) scaffolds and varied from 500 μm to 1100 μm for gradient (G) scaffolds. The fiber spacing was changed every millimeter. The scaffolds were plotted in blocks of 20×20 mm and 4 mm in height. The tested samples were 4×4 mm cylinders punched out from the blocks.

2.2. Cell expansion and culture

hMSCs (male, age 22) were retrieved from the Institute of Regenerative Medicine (Temple, Texas). Briefly, a bone marrow aspirate was drawn and mononuclear cells were separated using density centrifugation. The cells were plated to obtain adherent hMSCs, which were harvested when cells reached 60–80% confluence. These were considered passage 0 (P0) cells. These P0 cells were expanded, harvested and frozen at passage 1 (P1) for distribution. Cells were grown in MSC proliferation medium, which contains minimal essential medium (α-MEM, Life Technologies, Bleiswijk, the Netherlands) supplemented with 10% fetal bovine serum (FBS, Lonza), 100 U/ml penicillin (Life Technologies, Bleiswijk, the Netherlands), 10 μg/ml streptomycin (Life Technologies, Bleiswijk, the Netherlands), 2 mM L-glutamin (Life Technologies, Bleiswijk, the Netherlands), 0.2 mM L-ascorbic acid 2-phosphate magnesium salt (ASAp, Sigma-Aldrich, Zwijndrecht, The Netherlands) and 1 ng/ml of basic fibroblast growth factor-2 (bFGF-2, Fisher Scientific, Landsmeer, the Netherlands) at 37 °C in a humid atmosphere with 5% CO_2. Cells were expanded up to approximately 80% confluency and either frozen for further use or seeded on the scaffolds.

2.3. Cell seeding on scaffolds

After trypsinization with 0.25% trypsin (Life Technologies, Bleiswijk, the Netherlands), cells (passage 2–4) were counted using a Bückner chamber and re-suspended in proliferation medium at a density of 500,000 cells in 40 μL. The day before seeding, scaffolds were disinfected in 70% EtOH for 30 min under stirring, washed 3 times in phosphate buffered saline solution (PBS) (Lonza, Breda, the Netherlands), and incubated overnight in proliferation medium to allow protein adsorption on the scaffold's fibers. After protein adsorption, the 40 μL of cell suspension were placed on the scaffold in a drop wise fashion to account for a cell seeding density of 500,000 cells/scaffold. The seeded scaffolds were placed for 4 h in the incubator to allow cell adhesion before adding the cell culture medium.

Cells were cultured on the G and NG scaffolds for 7 days in proliferation medium. At day 7, the proliferation medium was changed and the cell-seeded scaffolds were cultured for another 28 days in basic medium presenting the same formulation as the proliferation medium without bFGF, or chondrogenic medium consisting of DMEM supplemented with 50 mg/mL ITS-premix (Bexton Dickinson), 0.4 mM Proline (Sigma-Aldrich, Zwijndrecht, The Netherlands), 50 mg/mL ascorbic acid (ASAp, Sigma-Aldrich, Zwijndrecht, The Netherlands), 100 mg/mL sodium pyruvate

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