Experimental Cell Research 357 (2017) 222–235

Contents lists available at ScienceDirect

Experimental Cell Research

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

Engineered cartilage regeneration from adipose tissue derived-mesenchymal stem cells: A morphomolecular study on osteoblast, chondrocyte and apoptosis evaluation

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ARTICLE INFO

Keywords:
Adipose tissue
Apoptosis
Cartilage
Chondrocytes
Collagen cell carrier scaffold
Lubricin
Mesenchymal stem cells
Regeneration
RUNX2

ABSTRACT

The poor self-repair capacity of cartilage tissue in degenerative conditions, such as osteoarthritis (OA), has prompted the development of a variety of therapeutic approaches, such as cellular therapies and tissue engineering based on the use of mesenchymal stem cells (MSCs). The aim of this study is to demonstrate, for the first time, that the chondrocytes differentiated from rat adipose tissue derived-MSCs (AMSCs), are able to constitute a morphologically and biochemically healthy hyaline cartilage after 6 weeks of culture on a Collagen Cell Carrier (CCC) scaffold. In this study we evaluated the expression of some osteoblasts (Runt-related transcription factor 2 (RUNX2) and osteocalcin), chondrocytes (collagen I, II and lubricin) and apoptosis (caspase-3) biomarkers in undifferentiated AMSCs, differentiated AMSCs in chondrocytes cultured in monolayer and AMSCs-derived chondrocytes seeded on CCC scaffolds, by different techniques such as immunohistochemistry, ELISA, Western blot and gene expression analyses. Our results showed the increased expression of collagen II and lubricin in AMSCs-derived chondrocytes cultured on CCC scaffolds, whereas the expression of collagen I, RUNX2, osteocalcin and caspase-3 resulted decreased, when compared to the controls. In conclusion, this innovative basic study could be a possible key for future therapeutic strategies for articular cartilage restoration through the use of CCC scaffolds, to reduce the morbidity from acute cartilage injuries and degenerative joint diseases.

1. Introduction

Articular cartilage (AC) is a specialized connective tissue that covers joint surfaces and facilitates the transmission of loads with a low frictional coefficient, allowing friction-free movement. Nevertheless, AC has very poor healing potential and is prone to both acute injury and degenerative conditions, such as OA, leading to severe histopathological changes and to an increased apoptosis rate and a decreased lubricin expression within the AC [1,2]. MSCs offer an option as a renewable source of cells and tissues, to be used in many diseases related to AC such as OA. The poor self-repair capacity of cartilage tissue has prompted the development of a variety of therapeutic

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http://dx.doi.org/10.1016/j.yexcr.2017.05.018
Received 2 March 2017; Received in revised form 23 April 2017; Accepted 18 May 2017
Available online 18 May 2017

approaches, such as tissue engineering and cellular therapies [3,4]. Tissue engineering is a part of biomedicine in which the knowledge of function of cells enables to control and promote the repair of damaged and diseased tissues. Cellular therapies could be an option aimed to improve the patient quality of life. Currently, autologous cell therapies such as autologous chondrocyte implantation (ACI) and matrix assisted ACI (MACI) require tissue to be explanted from a healthy area of cartilage, either from an intact portion of the damaged joint or from another of the patient joints. ACI is considered a promising therapeutic option for cartilage repair, however, allogeneic (unrelated cell donor) cell therapy has significant advantages over ACI including no donor site morbidity, healthy donors and reduced costs [5]. We have recently examined a potential allogeneic cell type for the treatment of OA, namely the chondrocyte-differentiated from AMSCs [6,7]. AMSCs are an adult stromal cell population possessing potent differentiation capacity into chondrocytes and osteocytes, representing a resource for use across major histocompatibility complex (MHC) barriers [8,9]. The involved procedures are less invasive and destructive than those used in AC specimens [10]. The differentiation of AMSCs into different cell types, in this case into chondrocytes, depends on the local microenvironment, growth factors, extracellular matrix and mechanical forces [11–13]. Cell death with morphological and molecular features of apoptosis has been detected in OA cartilage, suggesting a key role for chondrocyte death/survival in the pathogenesis of the disease. The identification of biomarkers of chondrocyte apoptosis may facilitate the development of novel therapies that may eliminate the cause of, or, at least, slow down the degenerative processes in OA [14]. Apoptosis is defined as physiologic cell death in order to remove harmful, damaged or unwanted cells without inducing an inflammatory response and without the release of cell contents as observed during necrotic cell death [15]. The two main mechanisms regulating apoptosis include the intrinsic pathway mediated by mitochondria and the extrinsic pathway [16] induced by death signaling ligands, such as tumor necrosis factor-alpha (TNFα) or FasL. These ligands bind their receptor and induce the initiator protease caspase-8, which in turn activates executioner proteases such as caspase-3. The caspase cascade plays a vital role in the induction, transduction, amplification and execution of apoptotic signals within the cell [17].

Lubricin is a chondroprotective mucinous glycoprotein that plays an important role in joint lubrication and synovial homeostasis, preventing cartilage wear and synovial cell adhesion [18,19]. As the goal of today's medicine shifts more and more towards disease prevention rather than treatment, lubricin might represent an attractive candidate therapy target molecule in the context of OA [20,21]. This important protein is found in several joint tissues including synovial membranes and fluid [22], the superficial zone of AC [23,24], tendons [25], ligaments [26], discs [27,28] and menisci [29,30]. Thanks to its boundary lubricating properties, lubricin prevents synoviocyte overgrowth, protects cartilage surfaces and prevents cartilage wear. Lubricin was also studied in an in vitro study directly on cells and in combination with artificial three-dimensional scaffolds [31].

In this study we obtain AMSCs from rat adipose tissue. In order to identify AMSCs derived from lipoaspirates, flow cytometry was carried out to identify specific cell surface markers of these cells (CD44, CD90 and CD105 positive; CD45, CD14 and CD34 negative). We differentiated AMSCs into chondrocytes through chondrogenic medium, after that the chondrocytes were seeded in a novel collagen scaffold for cell culture applications named CCC [32,33]. These scaffold samples were preconditioned in chondrogenic medium for 6 weeks. In this study we assessed the expression of some osteoblasts (runt-related transcription factor 2 (RUNX2) and osteocalcin), chondrocytes (collagen type I, collagen type II and lubricin) and apoptosis (caspase-3) biomarkers in undifferentiated AMSCs and in differentiated chondrocytes by ELISA, Western Blot and gene expression analyses. With the same molecules we evaluated also through immunohistochemical, biochemical and gene expression analyses the chondrocytes into the CCC scaffolds. These scaffolds were also morphologically examined.

The aim of this study is to demonstrate, morphologically and biochemically, for the first time, a healthy hyaline cartilage structure after 6 weeks of culture of differentiated chondrocytes from AMSCs, growing into CCC Scaffold, and the increased or decreased expression of lubricating ability through the study of lubricin and the apoptosis through the study of caspase-3. The presence or absence of lubricin and caspase-3 could be used as a biomarker match for the detection of chondrocyte's well-being and viability in CCC Scaffold indicating a healthy hyaline cartilage structure. Our study hypothesis is that these two molecules are inversely proportional and this data could help better understand this complex phenomenon for possible CCC scaffold implantation. Hopefully in the future the well-differentiated chondrocytes within CCC scaffolds could be used in therapeutic treatment to reduce the progression of cartilage degenerative diseases such as OA.

2. Materials and methods

2.1. Animals

Adipose tissue of subcutaneous fat was collected from twenty 10-month-old healthy female Wistar Outbred Rats (Charles River Laboratories, Milan, Italy), with an average body weight of 340 ± 60g undergoing liposuction procedures as previously described [34]. Rats were individually housed in polycarbonate cages at controlled temperature (20–23 °C) and humidity during the entire period of the study, with free access to water and food and a photoperiod of 12 h light/dark. The liposuction procedure was made under total anesthesia, 30 mg/Kg Zoletil 100+altadol 5 mg/kg+maintenance mixture of O_2 and isoflurane 2–2,5%, (Vibrac, Milan, Italia). Following anesthesia, the abdominal skin was shaved. We used a tumescent technique and a mixture (30–35 ml) of Ringer's lactate (Vibrac, Milan, Italia), lidocaine, adrenaline, and bicarbonate was injected into the rats undergoing liposuction. As in clinical practice, liposuction was performed using the syringe method. Instead of an aspirator, a 10-cc syringe connected to the cannula was used. Aspirated material (a mixture of fat tissue, blood, and serous fluid) was collected in the syringes and transferred into 15 ml sterile conical centrifuge tubes (Falcon, Milan, Italy) after the procedures. For liposuction, a small incision was made in the groin and a minimal dissection was made to facilitate aspiration of fat deposits in the subcutaneous region and to avoid trauma to the tissues. Liposuction was performed for 30 min on the right side and 30 min on the left side. The animals after liposuction procedures were sacrificed by intracardial Pentothal® injection 30–40 mg/kg (Biochemie, Kundl, Austria); under Furane 2%®-narcosis (Abbott Laboratories, Maidenhead, Berks, UK). All procedures conformed to the guidelines of the Institutional Animal Care and Use Committee (I.A.C.U.C.) of the University of Catania (Protocol n. 125 of the 1 July 2011, Italian Ministry of Health). The experiments were conducted in accordance with the European Community Council Directive (86/609/EEC) and the Italian Animal Protection Law (116/1992).

2.2. Culture of MSCs from adipose tissue

The lipoaspirate (a mixture of fat tissue, blood, and serous fluid) from each rat (1–5 ml) was washed with sterile PBS (Invitrogen, Milan, Italy) to remove red blood cells and debris, incubated for 3 h at 37 °C with an equal volume of serum-free DMEM low glucose (DMEM-1 g; Lonza, Milan, Italy) containing 0.075% of type I collagenase (Invitrogen, Milan, Italy). Collagenase was then inactivated by an equal volume of DMEM-1 g containing 10% of fetal bovine serum (FBS; Lonza, Milan, Italy). Subsequently, the digested lipoaspirate was centrifuged at 1200*g* for 10 min. The 20 pellets from each donor (rats) were divided in 40 flasks (2 flasks for each donor) and resuspended in PBS and filtered through a 100 μm nylon cell strainer (Falcon BD