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Extracellular matrix protein production in human adipose-derived mesenchymal stem cells on three-dimensional polycaprolactone (PCL) scaffolds responds to GDF5 or FGF2

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ABSTRACT

The poor healing potential of intra-articular ligament injuries drives a need for the development of novel, viable 'neo-ligament' alternatives. Ex vivo approaches combining stem cell engineering, 3-dimensional biocompatible scaffold design and enhancement of biological and biomechanical functionality via the introduction of key growth factors and morphogens, represent a promising solution to ligament regeneration. We investigated growth, differentiation and extracellular matrix (ECM) protein production of human adipose-derived mesenchymal stem/stromal cells (MSCs), cultured in 5% human platelet lysate (PL) and seeded on three-dimensional polycaprolactone (PCL) scaffolds, in response to the connective-tissue related ligands fibroblast growth factor 2 (basic) (FGF2) and growth and differentiation factor-5 (GDF5). Phenotypic alterations of MSCs under different biological conditions were examined using cell viability assays, real time qPCR analysis of total RNA, as well as immunofluorescence microscopy. Phenotypic conversion of MSCs into ECM producing fibroblastic cells proceeds spontaneously in the presence of human platelet lysate. Administration of FGF2 and/or GDF5 enhances production of mRNAs for several ECM proteins including Collagen types I and III, as well as Tenomodulin (e.g., COL1A1, TNMD), but not Tenascin-C (TNC). Differences in the in situ deposition of ECM proteins Collagen type III and Tenascin-C were validated by immunofluorescence microscopy. Treatment of MSCs with FGF2 and GDF5 was not synergistic and occasionally antagonistic for ECM production. Our results suggest that GDF5 alone enhances the conversion of MSCs to fibroblastic cells possessing a phenotype consistent with that of connective-tissue fibroblasts.

1. Introduction

Intra-articular ligament ruptures are a common musculoskeletal injury (Cheng et al., 2014) leading to painful joint instability, recurrent chondral injury, and disability, and early onset osteoarthritis (Watson and Ballet, 1984; Barrack et al., 1990; Petrigliano et al., 2006). Intracapsular ligaments, including the anterior cruciate ligament (ACL) and scapholunate (SL) ligaments, have an extremely limited intrinsic

Abbreviations: 3D, three-dimensional; ACL, anterior cruciate ligament; MSC, mesenchymal stem/stromal cells; calcein AM, calcein acetoxymethyl; ECM, extracellular matrix; EthD-1, ethidium homodimer; FGF2, fibroblast growth factor 2 (basic); GDF5, growth and differentiation factor-5; MSC, mesenchymal stromal/stem cell; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); PCL, polycaprolactone; PL, platelet lysate; RT-qPCR, real time reverse transcriptase quantitative polymerase chain reaction; SL, scapholunate

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ability to heal, stemming from its envelopment by synovial fluid and low vascularity (Watson and Ballet, 1984; Kimura et al., 2008). Due to this poor healing capacity, ligament injuries are usually treated with complete surgical reconstruction/replacement using allograft or autograft tendons. While this technique provides a significant improvement in clinical outcomes compared with direct ligament repair, the replacement of stiff ligaments with elastic tendon grafts has a number of negative long term consequences, including donor site morbidity, incomplete ligamentization, prolonged healing times and unfavorable immunogenic response (when allograft tendons are used) (Scheffler et al., 2008; Attia et al., 2014; Laurencin and Freeman, 2005). As a result, there has been a shift in focus to create neoligament tissues which can better mimic stable, functionally normal tissue. A variety of tissue-engineering strategies have been employed to achieve the requisite biomechanical properties. These include: i) decellularization of autografts, and the use of either ii) natural polymer or iii) synthetic polymer scaffolds. A-cellular scaffolds can be designed to meet the mechanical demands within the joint, but lack cellular conductive and inductive properties (Wagner et al., 2015). The combination of these scaffold structures with progenitor cells and growth factors, however, represents a promising solution to native ligamentous regeneration (Butler et al., 2000).

Mesenchymal stromal/stem cells (MSCs) are pluripotent progenitor cells capable of differentiating into a variety of musculoskeletal tissue precursors such as osteoblasts, chondrocytes, adipocytes, and myocytes when placed under specific conditions in vitro (Khan and Hardingham, 2012). Growth factors, hormones, and other regulatory molecules usually are incorporated into media to foster the differentiation of MSCs along specific lineages. Some physical factors including mechanical loading, electromagnetic fields, and ultrasound have been shown to play important roles in regulating the differentiation of MSCs (Xu et al., 2013; Naruse et al., 2000; Ziros et al., 2002).

Bone marrow stromal cells are among the best characterized stem cells and have been transplanted to various tissue injury sites, with enhanced tissue repair being achieved. However, there are drawbacks to their use. Bone-marrow-harvesting procedures are highly invasive, painful procedures with reported complication rates as high as 30% (Sasso et al., 2005). It is also well known that bone marrow isolations often yield a low number of stem cells (Varma et al., 2007). Due to the similar multipotential properties, adipose-derived mesenchymal stromal/stem cells (MSCs) have become a focus of research in recent years and studies demonstrated that MSCs could differentiate into lineages of multiple mesodermal tissues, such as bone, cartilage, fat, and muscle when under the appropriate conditions and provided key environmental cues (Petrigliano et al., 2006; Alexeev et al., 2014; Shi et al., 2012; Chen et al., 2006; Rodriguez et al., 2005). Typically, adipose tissue is abundant in both humans and animals and can be easily harvested from subcutaneous tissue through percutaneous or limited open aspiration techniques (Han et al., 2014; Oedayrajsingh-Varma et al., 2006). Adipose also provides a large volume of viable pluripotent stromal cells when harvested compared to that of bone marrow (Zuk et al., 2002). The ability to harvest such a high yield of stromal cells from small fractions of fat could mean that patients with low percentages of body fat could serve as autogenic sources of precursor cells for their own tissue-engineered therapies.

Fetal bovine serum (FBS) is mostly used for culture of human mesenchymal stem cells (hMSCs). However, translation of hMSC-based approaches is impeded by protracted expansion times, risk of xenogenic response, and exposure to zoonoses (Bieback, 2013). Human platelet lysate adherent to good manufacturing practices (GMP-hPL) provide a nonzoonotic adjuvant that enhances proliferation of hMSCs. Previous studies have shown that long-term culture in GMP-hPL maintains the multipotency of hMSCs, while protecting against clonal chromosomal instability detected in the FBS milieu (Crespo-Diaz et al., 2011). Thus, we use GMP-hPL, instead of FBS, to accelerate proliferation (with no chromosomal aberrancy) of cultured hMSCs when creating neoligaments.

Polycaprolactone (PCL) is a highly biocompatible aliphatic polyester obtained by the polymerization to open-loop of Ɛ-caprolactone with approval from the Food and Drug Administration (FDA) for use as an implantable material. It has excellent mechanical properties and exhibits slow degradation; it might therefore be a good candidate for use in in vivo tissue transplantation applications (Marino et al., 2012; Probst et al., 2010; Lam et al., 2009; Hutmacher et al., 2007; Schantz et al., 2006). We thus investigated growth, differentiation and gene expression and extracellular matrix (ECM) protein production of human MSCs seeded on three-dimensional polycaprolactone (3D PCL) scaffolds, both in the presence of platelet lysate (PL) alone, as well as in response to the connective-tissue related ligands basic fibroblast growth factor 2 (FGF2) and/or growth/differentiation factor 5 (GDF5). FGF2 has been considered for enhancement of tendon healing in vivo (Kraus et al., 2016; Tang et al., 2016; Oryan and Moshiri, 2014; Heisterbach et al., 2012; Thomopoulos et al., 2010a) and proliferation of MSCs for tendon engineering applications in vitro (Kimura et al., 2008; Durgam et al., 2012; Raghavan et al., 2012; Ker et al., 2011; Sahoo et al., 2010; Thomopoulos et al., 2010b; Reed and Johnson, 2014). GDF5 is known to be involved in tendon development in vivo (Tan et al., 2015; Mienaltowski et al., 2014; Hasslund et al., 2014; Dines et al., 2011) and has been investigated as a stimulatory morphogen for promoting tenogenic differentiation of mesenchymal stromal cells in culture (Wolfman et al., 1997; Mikic et al., 2001; Park et al., 2010). We therefore set out to establish whether human MSCs are capable of undergoing ligamentous differentiation on 3D scaffolds and whether exposure to FGF2, GDF5 or a combination of these treatments augments de novo formation of ligament tissue in vitro.

Our results suggest that GDF5 does enhance the conversion of human MSCs to fibroblastic cells, resulting in a phenotype consistent with that of connective-tissue fibroblasts. When compared to FGF2, GDF5 is more effective in stimulating this phenotypic conversion of MSCs to fibroblasts capable of producing high levels of ECM proteins on 3D PCL scaffolds.

2. Materials and methods

2.1. Human MSCs isolation and culture in vitro

Adipose-derived mesenchymal stem cells (MSC) were isolated as previously described (Moreau et al., 2005), in compliance with the institutional review board (IRB). Briefly, human adipose tissue was obtained from patients undergoing general surgical operations. The tissue was subsequently minced with scalpels, incubated in 0.075% collagenase type I (Worthington Biochemical, Lakewood, NJ) for 90 min at 37 °C, centrifuged at 500*g* for 10 min and passed through a 70 μm cell strainer (BD Biosciences, San Jose, CA). Expansion medium consisted of advanced Modification of Eagle's Media (aMEM) (Invitrogen, Carlsbad, CA) with 5% human platelet lysate adherent to good manufacturing practices (GMP-hPL) (Mill Creek, Rochester, USA), 2 U/mL heparin, 100 U/mL penicillin and 2 mM L-glutamine. Cultured cells were maintained in a humidified incubator at 37 °C and 5% CO_2. Medium was changed every 3 days, and cells were split at 70–80% confluency. All experiments were performed with cells from passage 6 and 7.

2.2. Seeding human MSCs on 3D PCL scaffolds

Three-dimensional polycaprolactone (PCL) scaffolds were acquired from Sigma-Aldrich Company (St. Louis, MO, USA). Fiber diameter is controlled by nozzle aperture while spacing between fibers is controlled by a motion control system, with a final configuration of 300 μm fiber diameter and 300 μm pore size. Cell seeding on 3D PCL scaffolds was performed according to the manufacturer 3D Insert™ cell seeding protocol, pipetting 150 μL MSC suspensions, with cell concentrations

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