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Fibrin hydrogels functionalized with cartilage extracellular matrix and incorporating freshly isolated stromal cells as an injectable for cartilage regeneration

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ABSTRACT

Freshly isolated stromal cells can potentially be used as an alternative to *in vitro* expanded cells in regenerative medicine. Their use requires the development of bioactive hydrogels or scaffolds which provide an environment to enhance their proliferation and tissue-specific differentiation *in vivo*. The goal of the current study was to develop an injectable fibrin hydrogel functionalized with cartilage ECM microparticles and transforming growth factor (TGF)-β3 as a putative therapeutic for articular cartilage regeneration. ECM microparticles were produced by cryomilling and freeze-drying porcine articular cartilage. Up to 2% (w/v) ECM could be incorporated into fibrin without detrimentally affecting its capacity to form stable hydrogels. To access the chondroinductivity of cartilage ECM, we compared chondrogenesis of infrapatellar fat pad-derived stem cells in fibrin hydrogels functionalized with either particulated ECM or control gelatin microspheres. Cartilage ECM particles could be used to control the delivery of TGF-β3 to IFP-derived stem cells within fibrin hydrogels *in vitro*, and furthermore, led to higher levels of sulphated glycosaminoglycan (sGAG) and collagen accumulation compared to control constructs loaded with gelatin microspheres. *In vivo*, freshly isolated stromal cells generated a more cartilage-like tissue within fibrin hydrogels functionalized with cartilage ECM particles compared to the control gelatin loaded constructs. These tissues stained strongly for type II collagen and contained higher levels of sGAGs. These results support the use of fibrin hydrogels functionalized with cartilage ECM components in single-stage, cell-based therapies for joint regeneration.

Statement of Significance

An alternative to the use of *in vitro* expanded cells in regenerative medicine is the use of freshly isolated stromal cells, where a bioactive scaffold or hydrogel is used to provide an environment that enhances their proliferation and tissue-specific differentiation *in vivo*. The objective of this study was to develop an injectable fibrin hydrogel functionalized with cartilage ECM micro-particles and the growth factor TGF-β3 as a therapeutic for articular cartilage regeneration. This study demonstrates that freshly isolated stromal cells generate cartilage tissue *in vivo* when incorporated into such a fibrin hydrogels functionalized with cartilage ECM particles. These findings open up new possibilities for in-theatre, single-stage, cell-based therapies for joint regeneration.

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1. Introduction

Articular cartilage is an avascular tissue with a complex structure that has a limited capacity for self repair [1]. Regenerating articular cartilage remains a challenge in the field of tissue engineering [1,2]. Chondral and osteochondral lesions often result in

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pain and swelling, followed by further joint degeneration and osteoarthritis [1,2]. Injuries to cartilage can be treated with a range of approaches, including marrow stimulating techniques, mosaicplasty and cell-based therapies [1]. Autologous chondrocyte implantation (ACI) and matrix-induced ACI (MACI) are examples of currently used techniques in articular cartilage regeneration, which have proved to be reasonably successful clinically [1,3]. However, these procedures require two surgeries and are significantly more expensive than traditional approaches to articular cartilage repair [4].

A number of different single-stage, cell-based procedures have been proposed that would theoretically overcome the need for two surgical procedures and autologous cell expansion [4–6]. Freshly isolated cells can potentially be obtained from harvested tissue in the surgical room, and efficiently implanted in one single procedure [5,7,8]. Freshly isolated stromal cells from the infrapatellar fat pad (IFP), which we have previously shown to be a viable source of chondro-progenitor cells [9–12], are a particularly promising cell type for single-stage procedures. In addition to identifying a suitable cell type, the successful realization of such in-theatre procedures also requires the development of a bioactive scaffold or hydrogel capable of promoting the proliferation and chondrogenic differentiation of a limited number of multipotent cells, which can be obtained from a donor in one surgical intervention.

Fibrin is a commonly used biomaterial for tissue engineering and is in widespread clinical use, typically as a hemostatic and/or a sealant agent [13]. It has also been investigated as a cell vehicle and as a therapeutic drug delivery system for different tissue engineering applications [13–15]. In the context of articular cartilage tissue engineering, there is evidence to suggest that fibrin is not as chondro-permissive as other well established hydrogels [16], with bone marrow and adipose-derived stem cells showing a diminished chondrogenic potential when encapsulated in fibrin [15–17]. There is therefore a clear need for further functionalization of this versatile injectable hydrogel system to optimize its utility for cartilage repair therapies.

Articular cartilage extracellular matrix (ECM)-derived materials have been previously used to engineer cartilage grafts with promising results [11,12,18,19]. In addition, such ECM-derived biomaterials have been used to bind and release chondrogenic factors such as transforming growth factor (TGF)-β3 [11,12]. Furthermore, ECM particles have also been used to functionalize other biomaterials in an attempt to enhance chondrogenesis [20–22]. For example, fibrin has been combined with ECM particles to develop implants for focal cartilage defect repair [23]. The use of such injectable hydrogels can also overcome limitations associated with pre-formed scaffolds, including challenges associated with fixation to complex cartilage defects and poor retention of newly synthesized ECM [24]. Therefore, such ECM functionalized fibrin hydrogels could potentially be used as an injectable carrier for freshly isolated stromal cells, with such a construct forming the basis of a single-stage therapy for articular cartilage regeneration. Hence, the objective of this study was to functionalize fibrin hydrogels with particulated cartilage ECM, and to assess the capacity of this construct to promote chondrogenesis of freshly isolated stromal cells *in vivo*. As promoting robust chondrogenesis *in vivo* may also necessitate exogenous growth factor presentation, this study also assessed the capacity of particulated cartilage ECM components to act as a controlled delivery system for TGF-β3 within a fibrin hydrogel. The chondro-inductivity of these cartilage ECM components was then compared to gelatin microspheres. These specific strategies were evaluated both *in vitro* and *in vivo* with the aim of assessing their potential as putative single-stage therapies for cartilage repair.

2. Material and methods

2.1. Preparation of particulated cartilage ECM

Articular cartilage for the fabrication of ECM particles was obtained from the articular joint of pigs (female, 3 months old), as presented in published work [12]. Briefly, cartilage was fragmented, and further fine ECM microparticles were fabricated by pulverizing/devitalizing the cartilage ECM in pieces with a cryogenic mill (6770 Freezer-Mill, SPEX). The ECM-derived particles were lyophilized (FreeZone-Triad, Labconco, USA), physically crosslinked and sterilized overnight using dehydrothermal treatment at 110 °C under vacuum.

2.2. SEM imaging of ECM-derived particles

ECM microparticles were observed and analyzed using scanning electron microscopy (SEM). Particles were fixed in 4% paraformaldehyde solution overnight. Microparticles were dehydrated through successive graded ethanol baths (10–100%), fixed in aluminium stubs, coated with gold and examined under a field emission scanning electron microscope (Tescan Mira FEG-SEM XMU, Libušina, Czech Republic). Images were analyzed with Image J to quantify microparticle size.

2.3. Fabrication of fibrin/ECM hydrogels

Fibrin hydrogels were produced using a method previously described [14,15]. Briefly, fibrin hydrogel constructs were fabricated by dissolving 100 mg/ml bovine fibrinogen (Sigma–Aldrich) in 10,000 KIU/ml aprotinin (Nordic Pharma, Sweden) containing 19 mg/ml sodium chloride (NaCl). Solution of Thrombin (5 U/ml) was made in 40 mM calcium chloride and adjusted to pH 7.0. Moreover, the optimal percentage loading of ECM particles in fibrin constructs was assessed based on literature [21,25] and additional experimental work. ECM was mixed with fibrinogen (fibrin) solution in 2% (w/v) and 10% (w/v) based on similar approaches [21,25]. An additional fibrin only was also prepared to serve as a control group. Fibrin/ECM or fibrin only solutions were mixed at a ratio of 1:1 with a 5 U/ml thrombin in 40 mM $CaCl_2$ solution and allowed to gel at 37 °C for 30 min yielding a final concentration of 50 mg/ml fibrin, 2.5 U/ml thrombin, 5000 KIU/ml aprotinin, 17 mg/ml NaCl and 20 mM $CaCl_2$ [14,15]. The final acellular hydrogels were 60 μl and were produced by using cylindrical agarose moulds (3% w/v; Sigma–Aldrich, Ireland), 5 mm in diameter. Furthermore, these fibrin/ECM hydrogels were scaled up for assessing the scalability of the method. The fibrin only, fibrin/ECM 2% and 10% (w/v) acellular hydrogels were prepared by using a 1.5 ml eppendorf tube as a mould.

2.4. Fabrication of gelatin microspheres and fibrin/gelatin hydrogels

Fibrin/gelatin hydrogels were produced using a previously described protocol [14], using a method similar to fibrin/ECM. However, in this particular case, gelatin microspheres [14,26,27] were used and incorporated into the fibrin hydrogel as previously described [14]. Briefly, microspheres were produced by a water-in-oil emulsion method. Gelatin was dissolved in deionised water and added drop-by-drop to 100 ml of olive oil heated to 45 °C while being continuously stirred. Gelatin concentration of 11% (w/v) was used in this study. After 10 min, the solution was cooled with additional stirring for 30 min, after which 40 ml of acetone was added and left for 1 h. Formed gelatin microspheres were collected through sieving (50 μm) and repeated washings in acetone. Microspheres were next crosslinked in 100 ml of glutaraldehyde solution

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