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Critical seeding density improves the properties and translatability of self-assembling anatomically shaped knee menisci

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ABSTRACT

A recent development in the field of tissue engineering is the rise of all-biologic, scaffold-free engineered tissues. Since these biomaterials rely primarily upon cells, investigation of initial seeding densities constitutes a particularly relevant aim for tissue engineers. In this study, a scaffold-free method was used to create fibrocartilage in the shape of the rabbit knee meniscus. The objectives of this study were to: (i) determine the minimum seeding density, normalized by an area of 44 mm^2, necessary for the self-assembling process of fibrocartilage to occur; (ii) examine relevant biomechanical properties of engineered fibrocartilage, such as tensile and compressive stiffness and strength, and their relationship to seeding density; and (iii) identify a reduced, or optimal, number of cells needed to produce this biomaterial. It was found that a decreased initial seeding density, normalized by the area of the construct, produced superior mechanical and biochemical properties. Collagen per wet weight, glycosaminoglycans per wet weight, tensile properties and compressive properties were all significantly greater in the 5 million cells per construct group as compared to the historical 20 million cells per construct group. Scanning electron microscopy demonstrated that a lower seeding density results in a denser tissue. Additionally, the translational potential of the self-assembling process for tissue engineering was improved though this investigation, as fewer cells may be used in the future. The results of this study underscore the potential for critical seeding densities to be investigated when researching scaffold-free engineered tissues.

1. Introduction

Fibrocartilage, a connective tissue found in various parts of the human body, frequently experiences degeneration, leading to conditions as diverse as osteoarthritis, difficulty in eating and speaking, and back pain [1–3]. Fibrocartilaginous tissues, such as the knee meniscus, temporomandibular joint disc and intervertebral disc, are responsible for withstanding significant mechanical loads in vivo, by virtue of the biochemical content of their native extracellular matrix (ECM). In particular, the collagens of the native ECM are responsible for conferring tensile strength and stiffness to these tissues, and the glycosaminoglycans (GAGs) are thought to provide compressive strength and stiffness [4,5].

The knee meniscus represents a prime candidate for research in tissue replacement or regeneration. Meniscal lesions alone are responsible for nearly 1 million surgeries annually in the US and Europe, and are the root cause of the most frequent procedures practiced by orthopedic surgeons [5,6]. The knee meniscus displays a specific, functionally important wedge shape, as well as mechanical anisotropy between its circumferential and radial directions [7–9]. This very specific organization allows the knee meniscus to effectively transfer loading from the distal femur to the tibial plateau [10]. Thus, the biomechanical functions of the knee meniscus are inexorably tied to the structure of tissue. Importantly, the knee meniscus and all fibrocartilages display a fundamental inability to mount a healing response after structural damage is sustained due to trauma, aging or disease, and thus represent ideal candidates for replacement by tissue engineering [10,11].

Recently, our laboratory has pioneered a scaffoldless approach, termed the self-assembling process in tissue engineering, which relies upon cadherin-mediated biophysical interactions that occur early during seeding of cells in a high-density culture [12]. Similar studies, focused on engineering scaffold-free liver and optic tissues, have also reported all-biologic constructs displaying the presence of cadherins early during tissue culture [13,14]. In the self-assembling process specifically, the use of a non-adherent seeding well and the up-regulation of N-cadherin early during

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culture result in the minimization of free energy during tissue formation [15,16]. Despite the fact that cells are not seeded within a biomaterial, the non-adherent well serves as a mold during this process, providing support to the developing neotissue and helping determine its final shape. This approach, first demonstrated for articular cartilage, has also been extended to the generation of fibrocartilage [17]. In particular, this has resulted in success via the creation of all-biologic constructs unhindered by intervening synthetic scaffolds [18–21]. Additionally, self-assembling constructs may lack the immunogenicity and potential toxic degradation byproducts of some biomaterial scaffolds. Finally, self-assembling constructs could avoid stress-shielding in vivo and have the potential for more seamless integration with native tissue [16]. However, despite these successes, additional investigations are necessary to understand and enhance structure–function relationships involving ECM content/organization, mechanical properties, and construct size and geometry.

The number of cells needed to form a construct is a non-trivial issue in tissue engineering and regenerative medicine. While one may infer that greater cell numbers may imply a larger tissue construct, this is not always the case [22,23], and there is no guarantee that the properties of engineered tissue constructs scale linearly as the construct size increases. Furthermore, several musculoskeletal tissues, including fibrocartilage, possess relatively low cellularity. From a translational perspective, generating a tissue construct requires a large number of cells, whose acquisition from a patient or donor may or may not be feasible, especially if an investigator is employing an autologous or allogeneic approach. Interestingly, altering the seeding density has repeatedly been reported to change or improve the quality of tissue constructs [24–26]. Recent work in our laboratory has demonstrated the use of this for self-assembling articular cartilage [23], although the generation of shape-specific knee meniscus fibrocartilage and the relationship of its biochemical and biomechanical properties to seeding density remain unknown.

The objectives of this study were to: (i) determine the minimum seeding density, normalized by an area of 44 mm^2, necessary for the self-assembling process of fibrocartilage to occur; (ii) examine relevant biomechanical properties of engineered fibrocartilage, such as tensile and compressive stiffness and strength, and their relationship to seeding density; and (iii) identify a reduced, or optimal, number of cells needed to produce this biomaterial. Self-assembling fibrocartilage constructs were seeded with varying numbers of cells in the shape of the native knee meniscus using non-adherent agarose molds of constant size and cultured for 4 weeks. At the end of culture, the construct properties were assessed. It was hypothesized that: (i) a threshold seeding density existed, below which the self-assembling process would not occur; (ii) construct biomechanical and biochemical properties would increase with greater seeding densities and eventually plateau; and (iii) constructs could be seeded with fewer cells than the historical density of 20 million per construct while possessing equivalent or greater biochemical and biomechanical properties.

2. Materials and methods

2.1. Cell isolation

Bovine articular chondrocytes and meniscal cells were harvested from the legs of four 8-week-old calves (Research 87) [27,28]. Chondrocytes were obtained from the entire surface of the distal femur, and meniscal cells were obtained from the meniscus after trimming out the outer meniscal rim. The tissues were minced into approximately 1 mm^3 pieces. Cartilage was digested in 0.2% (w/v) Worthington's "collagenase type II" enzyme mixture (Worthington) in base medium (Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 3% (v/v) fetal bovine serum (FBS; Benchmark), 1% (v/v) non-essential amino acids (Invitrogen) and 1% penicillin/streptomycin/fungizone (PSF; Lonza) for 18 h. Meniscal tissue was digested in a 0.25% (w/v) pronase (Sigma) in base medium for 1 h, followed by a 0.2% (w/v) collagenase type II (Worthington) in base medium for 18 h. After digestion, the tissues were washed and centrifuged four times using phosphate-buffered saline (PBS) and filtered through 70 μm cell strainers. Chondrocytes were frozen in DMEM with 20% (v/v) dimethylsulfoxide and 10% (v/v) FBS, and kept in liquid nitrogen until resuspension with freshly isolated meniscal cells for seeding, as described previously [12,17].

2.2. Construct seeding and sample preparation

To generate self-assembling fibrocartilage, cells were seeded in non-adherent meniscus-shaped agarose wells formed from a rapid prototyped (ZPrinter) wedge-shaped ring mold, as described previously [29]. Meniscus-shaped ring wells were fabricated with a major axis of 9.5 mm and a minor axis of 7 mm, and an inner post with a major axis of 4 mm and a minor axis of 2.5 mm. Wells had a sloped geometry, with an inner depth of 12.5 mm and an outer depth of 14 mm. The internal volume of each well was approximately 200 μl. The approximate area of the bottom surface of each meniscus-shaped construct was 44 mm^2 [30]. The initial seeding densities were 1.25, 2.5, 5, 10, 15 and 20 million cells per construct. Each construct was seeded within 180 μl of liquid while keeping the dimensions of the well constant and varying the total number of cells. To form each construct, chondrocytes and meniscal cells were seeded at a 1:1 ratio in 180 μl of a chondrogenic medium formulation described previously [17]. The chondrogenic medium formulation was as follows: DMEM with GlutaMAX (Invitrogen), 100 nM dexamethasone (Sigma), 1% (v/v) PSF (Lonza), 1% (v/v) ITS + premix (BD biosciences), 50 μg ml^{-1} ascorbate-2-phosphate (Sigma), 40 μg ml^{-1} L-proline and 100 μg ml^{-1} sodium pyruvate (Fischer Scientific). An additional 500 μl of medium was added 4 h after seeding. The total volume of medium in each well was changed daily, following an internal pilot study. The medium volume was 6 ml during the first week of culture and 2 ml thereafter. All cultures were kept at 37 °C with 10% (v/v) CO_2. After 1 week, tissue constructs were unconfined from their agarose wells and kept in free floating culture for an additional 3 weeks. During this time, constructs were cultured in a 24-well culture plate with 400 μl of agarose coated to the bottom of the well to prevent any potential adhesion of the constructs, or cells within the constructs, to the plate. After a total culture time of 4 weeks, the construct gross morphology and wet weight were assessed before division of each construct into pieces for mechanical, biochemical and histological analysis. Each construct was split in the same manner, with sections being taken from similar areas for each specific test. No heterogeneities were observed. All photographs of constructs for gross morphology, compressive testing and tensile testing were taken using a Canon 100 mm f/2.8 macro lens.

2.3. Histology

Whole constructs were frozen at −20 °C in HistoPrep (Fisher Scientific) after culture, and additional tissue constructs were taken at days 1, 4, 7, 14 and 22 for comparison. Sections were fixed in formalin prior to staining with Safranin-O/Fast Green for GAG content and Picrosirius Red for collagen content. Other sections were fixed in acetone for immunohistochemistry (IHC) staining of collagen I and collagen II. IHC was performed with the Vectastain ABC and DAB Substrate Kits (Vector Labs) with rabbit anti-human collagen I (US Biologics) and rabbit anti-bovine collagen II