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The role of organ level conditioning on the promotion of engineered heart valve tissue development *in-vitro* using mesenchymal stem cells

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ABSTRACT

We have previously shown that combined flexure and flow (CFF) augment engineered heart valve tissue formation using bone marrow-derived mesenchymal stem cells (MSC) seeded on polyglycolic acid (PGA)/poly-L-lactic acid (PLLA) blend nonwoven fibrous scaffolds (Engelmayr, et al., Biomaterials 2006; vol. 27 pp. 6083–95). In the present study, we sought to determine if these phenomena were reproducible at the organ level in a functional tri-leaflet valve. Tissue engineered valve constructs (TEVC) were fabricated using PGA/PLLA nonwoven fibrous scaffolds then seeded with MSCs. Tissue formation rates using both standard and augmented (using basic fibroblast growth factor [bFGF] and ascorbic acid-2-phosphate [AA2P]) media to enhance the overall production of collagen were evaluated, along with their relation to the local fluid flow fields. The resulting TEVCs were statically cultured for 3 weeks, followed by a 3 week dynamic culture period using our organ level bioreactor (Hildebrand et al., ABME, Vol. 32, pp. 1039–49, 2004) under approximated pulmonary artery conditions. Results indicated that supplemented media accelerated collagen formation (~185% increase in collagen mass/MSC compared to standard media), as well as increasing collagen mass production from 3.90 to 4.43 pg/cell/week from 3 to 6 weeks. Using augmented media, dynamic conditioning increased collagen mass production rate from 7.23 to 13.65 pg/cell/week (88.8%) during the dynamic culture period, along with greater preservation of net DNA. Moreover, when compared to our previous CFF study, organ level conditioning increased the collagen production rate from 4.76 to 6.42 pg/cell/week (35%). Newly conducted CFD studies of the CFF specimen flow patterns suggested that oscillatory surface shear stresses were surprisingly similar to a tri-leaflet valve. Overall, we found that the use of simulated pulmonary artery conditions resulted in substantially larger collagen mass production levels and rates found in our earlier CFF study. Moreover, given the fact that the scaffolds underwent modest strains (~7% max) during either CFF or physiological conditioning, the oscillatory surface shear stresses estimated in both studies may play a substantial role in eliciting MSC collagen production in the highly dynamic engineered heart valve fluid mechanical environment.

1. Introduction

Surgical replacement of diseased heart valves by mechanical and tissue valve substitutes is now commonplace and enhances the survival and quality of life for many patients. However, repairs of congenital deformities require very small prosthetic heart valve sizes that are not commercially available. Further, in pediatric applications which seek to correct congenital anomalies of the pulmonary valve, accommodation of somatic growth is essential to eliminate multiple reoperations. Tissue engineered pulmonary valves (TEPV) are one such approach that have the potential to develop into a permanent replacement [1]. A fundamental aspect of the development of a TEPV capable of physiologic function at the time of implant is knowledge of the biological and mechanical stimulatory requirements to form mechanically robust, biomimetic valvular tissues *in-vitro* [2,3].

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It has become axiomatic that *in vitro* mechanical conditioning promotes engineered tissue formation *in-vitro*. However, the underlying mechanisms remain largely unknown, as well as a means to systematically translate these phenomena to the organ level. This lack of knowledge remains a major limiting factor in the rational development engineered heart valve tissues, especially when considering that valve leaflets are subjected to complex, time varying loading environments [4–12]. Thus, many questions remain regarding the exact nature and timeframe of *in-vitro* conditioning necessary for optimal tissue formation. This optimization process must also take into consideration multiple factors, such as leaflet shape and scaffold material properties that will provide the construct with sufficient *in vivo* functionality. At a minimum, sufficient tissue formation, with the appropriate physical qualities, needs to occur prior to implantation so that the TEPV construct can functionally sustain the *in vivo* hemodynamic environment.

Previous work by Mol et al. [10] reported that enhanced tissue formation was observed in conditioned leaflets. Hoerstrup et al. [8] reported on the use of a pulse duplicator system to condition tissue engineered valves at ramping pressure conditions from 30 to 55 mm Hg over a 28 day period. They further reported on substantial increases in the net collagen and DNA content of their conditioned constructs over controls using primary myofibroblasts and endothelial cells [8,10]. However, the specific nature of fluid induced stresses acting on the leaflet surfaces in these studies remains unknown.

Native and prosthetic heart valves are subjected to highly complex, time-varying flexural, tensile, and fluid-induced stresses [13]. Clearly, studies utilizing continuous dynamic stimulation of TEPVs would provide insights into the coupled effects of these stresses during the *in-vitro* conditioning process. The need for such studies has thus far prompted several developments in the form of custom-made devices or bioreactors that can provide biomimetic mechanical conditioning in the heart valve tissue engineering context [4,7,10,14]. However, an optimal conditioning protocol, and the underlying models to develop them for valvular development, remains unknown. Such approaches are particularly important to develop bioreactor-based conditioning regimes using novel (potentially clinically feasible) cell sources [7,15–18] suitable for functional TEPV development.

Previous studies have explored the use of bone marrow derived mesenchymal stem cells (MSCs) as a potential autologous cell source for engineered heart valve tissues, since they can be obtained in a minimally invasive fashion [19]. Further, while yet to be comprehensively demonstrated, MSC pluripotency may allow them to serve as a clinically feasible cell source without raising ethical questions associated with embryonic stem cells. With respect to *in-vitro* tissue conditioning using MSCs, we and others demonstrated that they respond favorably to mechanical stimuli [20–24]. Sutherland et al. [18] also demonstrated that under extended implantation periods (>4 months), TEPVs in an ovine model using MSCs seeded onto polyglycolic acid (PGA)/poly-L-lactic acid (PLLA) nonwoven, biodegradable scaffolds underwent extensive remodeling leading to structures resembling the native valve.

Development of optimal *in vitro* conditioning regimes is dependent on a wide range of variables, including type/magnitude of stresses applied, leaflet motion and geometry, conditioning timeframes, cell source(s), scaffold materials, and media formulation, to name a few. All of these variables (and potentially many more) will require knowledge of their respective effects on both initial and subsequent post-implant tissue formation. Thus, in order to develop a rational, mechanism-based approach directed towards understanding the processes that occur during *in-vitro* engineered heart valve tissue development, our laboratory has focused on elucidating the relative contributions of the major modes of heart valve deformation: flow, flexure, and stretch, which are central to valve dynamics [19,25]. In particular, utilizing a specialized bioreactor we have demonstrated that combined cyclic flexure and flow (CFF) conditions result in significantly higher collagen content (~75% increase) in comparison to isolated flex and flow conditions alone [19]. Interestingly, in contrast to vascular smooth muscle cell-seeded scaffolds prepared under static and cyclic flexure-alone environments [5], MSC-seeded scaffolds accumulated less collagen [19]. While these studies have provided initial insight into the complexities of optimization of engineered heart valve tissue properties, the underlying mechanisms of how external fluidic forces and internal deformations modulate tissue formation remain unknown. In particular, the potential to scale-up the phenomenon observed in our CFF and related studies to the complex physical milieu of a functional tri-leaflet valve under physiological hemodynamic conditions remain unproven.

As a next step, we conducted the following study to determine if the extracellular matrix (ECM) growth patterns observed in our previous CFF studies [19] could be duplicated in a functional tri-leaflet valve. Specifically, we utilized ovine MSCs to seed nonwoven PGA/PLLA fabrics and imposed pulmonary artery conditions on intact tri-leaflet valve constructs and compared them to valves grown under static tissue culture conditions. The resulting formed tissues were studied using histological and biochemical measures. In addition, it has been shown that media containing ascorbic acid-2-phosphate (AA2P) and basic fibroblast growth factor (bFGF) augmented tissue formation [18]. Owing to the critical role of collagen in the functional requirements of engineered heart valve tissues [5,26–28], we assessed the coupled effects of ascorbic acid 2-phosphate (AA2P) and basic fibroblast growth factor (bFGF) on collagen and GAG formation. Next we combined these biochemical enhancements with physiological conditioning on tri-leaflet heart valve constructs to evaluate our ability to perform scale-up at the organ level. Finally, in an effort to gain insight into the potential stimulatory mechanisms of MSC seeded TEVC tissue formation, we developed detailed computational fluid dynamic (CFD) models of the CFF bioreactor specimens to specifically delineate the nature in which fluid-induced shear stresses act on CFF specimens, and related them to similar patterns reported for the native tri-leaflet valve.

2. Methods

2.1. Isolation and characterization of MSCs

Ovine MSC isolation was performed following a previously reported protocol by Pittenger et al. [29]. In brief, 10 ml/kg of whole bone marrow was aspirated from the iliac crest of neonatal sheep. Cells were centrifuged on a Ficoll gradient in order to obtain the mononuclear cell fraction. Red blood cells were lysed with ammonium chloride, and the remaining mononuclear fraction was plated onto bacteriologic plates. Medium was initially changed after 48 h, and adherent cells were passaged.

MSC characterization was performed using the methodology reported by Perry et al. [12]. Briefly, a sub-population of these cells were grown and plated at 10,000 cells/cm^2, and after initial culture in standard medium, cells were grown in media known to differentiate MSCs into fat cells (adipogenic medium), bone cells (osteogenic medium) or cartilage (chondrogenic medium). After three weeks, cells were found to differentiate into the three cell types. Subsequently, characterized MSCs were cryopreserved in culture medium supplemented with 5% dimethyl sulfoxide (DMSO) and were shipped to the University of Pittsburgh on dry ice.

2.2. MSC culture and AA2P and bFGF supplementation studies

MSCs were expanded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic plus HEPES buffer (all purchased from Invitrogen, Carlsbad, CA). Cells were at passage 8–10 when sufficient numbers were available for scaffold seeding. A needled nonwoven scaffold consisting of 50:50 blend of PGA and PLLA fibers (Concordia Fibers, Coventry, RI) was utilized as the scaffold for all experiments. The PGA/PLLA scaffolds had an initial fabric thickness of 1.53 mm and density of 59.8 mg/ml. Scaffolds were cut into 5 mm