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Evaluation of the matrix-synthesis potential of crosslinked hyaluronan gels for tissue engineering of aortic heart valves

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Abstract

Our goal is to fabricate continuous sheets of elastin atop non-biodegradable hydrogels (hylans) containing crosslinked hyaluronan, a glycosaminoglycan. Such elastin–hyaluronan composites may be useful to tissue engineer replacements for the glycosaminoglycan- and elastin-rich layers of the native aortic valve cusp. Neonatal rat aortic smooth muscle cells were cultured atop hylan gels with micro-textured surfaces, and on plastic, and the components of the extracellular matrix (collagen, elastin) were periodically analyzed.

The hylan substrates induced the cells to proliferate more rapidly and over longer time periods ($\sim$4 weeks) relative to those cultured on plastic (2–3 weeks). Consequently, at all assay times, the amounts of elastin was derived from the hylan-based cell cultures was 25% or more than that derived from cells cultured on plastic. However, when elastin content was normalized to the cell DNA content, no significant differences were found in the two substrates beyond the first two weeks of culture. Conversely, at culture times greater than 2 weeks, cells cultured atop hylan gels produced amounts of collagen/nanogram of DNA that were $\sim$56% less than that synthesized by cells cultured on plastic. Cells grown on hylan deposited an unusual matrix layer, rich in elastin, at the hylan-cell interface. This elastin was found to be organized into fenestrated sheets and loose elastin fibers, structures that were also isolated from the elastin matrix of the ventricularis layer of porcine aortic valve cusps. We have thus demonstrated that hylan gels are useful as substrates to induce elastin synthesis in culture to obtain structures that resemble the elastin matrix of the native aortic valve.
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1. Introduction

Tissue engineering (TE) is a promising approach for overcoming the deficiencies of currently available mechanical and bioprosthetic heart valves. Mechanical valves are rigid, require chronic anticoagulant therapy, and are at risk of sudden failure [1,2]. Bioprosthetic valves, on the other hand, have poor long-term durability due to tissue calcification and mechanical damage [3]. Tissue-engineered aortic valves could possibly overcome these problems. However, such valves present their own set of challenges. Recent studies with acellularized xenogeneic aortic valve implants in sheep have demonstrated some repopulation with host cells [4] but rapid and complete remodeling of the valve has not been observed. Thus, the conventional tissue engineering approach of seeding cells on a biodegradable scaffold, and expecting mature and functional tissue to regenerate in vivo as the scaffold degrades, is unlikely to be successful. A more promising approach would be to fabricate the valve microstructure in vitro using matrix molecules and cells [5], so that the tissue could be functional immediately upon implantation.

The unique mechanical characteristics of native aortic valve cusps are a result of their complex multilayered morphology [6]. The fibrosa, composed mainly of collagen bundles, is the primary load-bearing layer of the tissue. The ventricularis contains elastin sheets and

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fibers that keep the fibrosa in a compressed state [7,8]. The central spongiosa layer contains glycosaminoglycans (GAGs) that keep the cusp hydrated, and permits shear between the fibrosa and ventricularis during tissue loading and unloading. Our approach to developing a tissue engineered aortic valve is to integrate the above functional components into a composite valve cusp, after each has been fabricated separately in vitro.

A non-biodegradable hydrogel (hylan) fabricated by crosslinking hyaluronan (HA), a GAG with divinyl sulfone (DVS), was selected as a material for replacing the spongiosa layer of the heart valve cusp. The choice of material is appropriate since HA and Chondrotin sulfate compose roughly 90% of the total GAGs in human aortic heart valves [9,10]. Hyaluronan-based biomaterials are relatively biocompatible, and lowly antigenic and immunogenic because of the structural homology of the HA molecule across species [11]. Several methods have been documented previously for derivatizing and crosslinking HA to produce a variety of materials with widely different mechanical characteristics [12,13]. We selected DVS as a crosslinker because it produces a gel with appropriate viscous mechanical characteristics [14]. Moreover, since DVS crosslinking does not involve the biologically reactive functional groups on the HA molecule, the gels largely retain the biological properties of native HA, including their non-antigenicity, and non-immunogenicity [15–17]. Hylan gels however do not facilitate cell attachment and are thus inherently poor scaffolds for tissue engineering applications. To enhance cell attachment, we have previously developed a technique to micro-texture the gel surface by controlled exposure to UV light [18]. We demonstrated that neonatal rat aortic smooth muscle cells (NRASMC) attach readily to the UV-treated gels, and proliferate. In the current study, we have extended this work to evaluate the synthesis of structural extracellular matrix (ECM) molecules such as elastin and collagen, on the hylan gel substrates. The ultimate long-term objective is to use hylan gels as cell scaffolds for the in vitro synthesis of elastin and collagen matrices that resemble the complex matrix ultrastructure observed in native aortic valve cusps. A more immediate goal is to fabricate an elastin–hylan composite of the appropriate microstructural organization to replicate the mechanics of the spongiosa and ventricularis layers of the native aortic valve cusp.

The specific aims of this study are to (i) characterize the extracellular matrix qualitatively and quantitatively, (ii) evaluate hylan gels as substrates for the synthesis of an elastin matrix, and (iii) compare and contrast the microstructure of the cultured elastin to that present in the ventricularis layer of the native aortic valve cusp.

2. Materials and methods

All hylan gels used in this study had their surface texturized by irradiation with UV light under hydrated conditions, as per previously published protocols [18]. These modified hylan gels were then seeded with NRASMCs, selected primarily for their demonstrated ability to prolifically synthesize the ECM proteins collagen and elastin, in vitro [19]. The proliferation of cells seeded atop plastic or hylan gels was evaluated via a DNA assay at bi-weekly intervals, over 6 weeks of culture. Elastin and collagen in the extracellular matrix were quantified biochemically using the Fastin and Hydroxyproline (OH-Pro) assays respectively, at the same assay times. The presence of collagen and elastin within the cell layer was affirmed using immuno-fluorescence labeling techniques. Transmission electron microscopy (TEM) was used to investigate the ultrastructure of the synthesized extracellular matrix and trace the time-progression of elastin and collagen synthesis and maturation. Finally, the synthesized elastin matrix was visualized using scanning electron microscopy (SEM) to compare its three-dimensional structure to that of elastin matrices isolated from heart valve tissues. NIH guidelines for the care and use of laboratory animals were observed in this study (NIH Publication #85-23 Rev. 1985). The Biological Resources Unit at the Cleveland Clinic, where this study was performed, approved all animal procedures.

2.1. Formulation of hylan gels

The formulation of hylan gels used in this study was based on a modification of a previously patented protocol [20]. To prepare the gels, 330 mg of the sodium salt of long chain HA (Molecular weight $> 1.5 \times 10^6$) obtained as a sodium salt from Genzyme Biosurgicals (Cambridge, MA), was mixed with 1 M NaOH, pH 12.0 at 4°C. After mixing for 30 min, gelation was initiated by addition of 64 μl of DVS (Sigma Chemicals, St. Louis, MO). The DVS crosslinker was emulsified in 1 ml of 1 M NaCl (4°C) to obtain homogenous crosslinking within the mixture. The HA solution was immediately transferred using a syringe to 35-mm diameter wells in a 6-well plate and permitted to gel over 2 h at 23°C. Sodium hydroxide within the gels was removed via equilibration with an excess of distilled water. The gels were then rinsed with three changes of a 70:30 volume ratio of isopropanol and 1 M NaCl to leach out unreacted DVS and also limit swelling. Finally, in preparation for cell culture, the gels were equilibrated with a serum-free 1:1 volumetric mixture of Dulbecco's Modified Eagle's cell culture medium (DMEM) and Hams F-12 nutrient mixture (DMEM: F12; Life Technologies, Grand Island, NY). In all gels, the ratio

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