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Heparin stimulates elastogenesis: Application to silk-based vascular grafts

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ABSTRACT

With over 500,000 coronary artery bypass grafts (CABG) performed annually in the United States alone, there is a significant clinical need for a small diameter tissue engineered vascular graft. A principle goal in tissue engineering is to develop materials and growth conditions that encourage appropriate recellularization and extracellular matrix formation *in vivo*. A particular challenge in vascular engineering results from the inability of adult cells to produce elastin, as its expression is developmentally limited. We investigated factors to stimulate elastogenesis *in vitro*, and found that heparin treatment of adult human vascular smooth muscle cells promoted the formation of elastic fibers. This effect was heparin-specific, and dependent on cell density and growth state. We then applied this information to a silk-based construct, and found that immobilized heparin showed essentially identical biological effects to that of soluble heparin. These findings indicate that heparinized vascular grafts may promote elastin formation and regulate restenosis, in addition to heparin's well-established antithrombotic properties. Given the increase in elastin mRNA level and the increase in extracellular elastin present, our data suggests that there may be multiple levels of elastin regulation that are mediated by heparin treatment.

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1. Introduction

The high incidence of heart disease results in more than 500,000 coronary artery bypass grafts (CABGs) performed each year in the United States (AHA, 2007). In these procedures, a donor vessel is typically harvested from the patient's internal mammary artery or saphenous vein to use for transplant. Donor vessels are often unsuitable for transplant due to advanced age or illness. Vascular conduits made of Dacron or expanded polytetrafluoroethylene (ePTFE) are available for large diameter (>6 mm) grafts; however, these materials fail at smaller diameters due to thrombosis (Harris and Seikaly, 2002). This unmet need drives research into alternative tissue engineered vascular grafts for small diameter vessels.

A key challenge in vascular conduit engineering is the development of robust elastic laminae throughout the new matrix material upon repopulation with native cells and their deposited extracellular matrix (Mitchell and Niklason, 2003). Elastic laminae are critical for proper functioning of elastic arteries, which include the major coronary vessels. In addition to their mechanical properties, elastic laminae biochemically regulate SMC proliferation and migration (Karnik et al., 2003). Elastic fiber biology has been studied *in vitro* using fetal and neonatal mammalian cells, which has shed significant light on the assembly process. Current data indicate that initially, fibrillin-1 fibers are assembled and deposited extracellularly. Tropoelastin monomers are then deposited onto the fibrillin, in a process likely organized by fibulin-5 and other elastin associated proteins. Tropoelastin is then crosslinked by lysyl oxidase and related family members to form mature elastin fibers (Kielty, 2006). Though this process has been extensively studied, two challenges to inducing exogenous elastin generation are presented. First, properly organized elastin is not produced from adult smooth muscle cells, as its expression is developmentally limited and ceases after childhood (Davis, 1993; El-Hallous et al., 2007). As adult SMC in culture do not produce elastin, attempts to study differences in molecular organization, mechanical properties, or even to optimize elastin production between the adult-originating cells and fetal cells have been largely unsuccessful. (Swee et al., 1995; Kelleher et al., 2004)Thus, developing and characterizing a system to robustly promote elastin formation from adult tissues becomes a necessary first step. The lack of elastin deposition by adult SMCs significantly impacts the field of tissue engineering, as any biodegradable scaffold would ideally be populated with ECM-depositing cells. As elastin is a critical protein for mechanical properties of blood vessels, its deposition from SMCs is highly desirable. Though some elastin is formed in response to injury, this is generally not properly organized (Arribas et al., 2008). Second, the complex nature of the elastic fiber (due to its many accessory proteins) may be partly responsible for the inability of tropoelastin overexpression alone to form mechanically robust fibers (Katsuta et al., 2008).

Abbreviations: hAoSMC, human aortic smooth muscle cell; DTAF, dichlorotriazinylaminofluorescein; HAEC, human aortic endothelial cell; LbL, layer-by-layer.

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There have been several recent reports demonstrating direct biochemical interactions between heparin, tropoelastin and fibrillin (Cain et al., 2005; Tu and Weiss, 2008). Heparin is a glycosaminoglycan (GAG) composed of alternating glucosamine and uronic acid sugars, which are extensively sulfated to confer a high degree of negative charge (Mishra-Gorur and C., 1999). Native heparin ranges from 8,000–20,000 Da (approximately 30–70 sugar residues) in mass, whereas clinical grade low molecular weight heparin (LMW-heparin; tradenames Enoxaparin, Lovenox) is about 3,000 daltons (approximately 10 sugar residues). Heparan sulfate (HS) is a close biochemical relative of heparin, however HS covalently attached to one of many protein cores to form a heparin sulfate proteoglycan (HSPG). Additionally, HS consists of a lower percentage of iduronic acid residues, and is less sulfated than heparin, resulting in a more positive overall charge. Physiologically, heparin is stored in mast cells and is also secreted by endothelial cells, thereby forming part of the blood vessel extracellular matrix (Lever and Page, 2002). Heparin has both anticoagulant properties and antiproliferative properties, though these properties are independent as previously demonstrated (Castellot et al., 1984). These properties, in addition to our findings which show heparin-induced elastogenesis, make heparin addition an attractive biochemical modification for silk vascular grafts.

Silk derived from *Bombyx mori* silkworm cocoons is a useful biomaterial for vascular tissue engineering. Following initial processing to remove immunogenic sericin proteins, the liquid silk can be molded into vessels of any diameter, with varied porosity (Lovett et al., 2008). Silk is also nonimmunogenic, mechanically strong, biodegradable with controlled kinetics, and can be chemically modified (Altman GH et al., 2003).

We hypothesized, given the known antiproliferative and anticoagulant activities and the elastogenic potential of heparin described herein, that heparin contained in silk films would increase organized elastic fibers from SMCs cultured on these films. This would provide a foundation for a silk-based heparin delivery system for vascular graft applications. Indeed, here we present evidence of regulated smooth muscle cell response to heparinized silk constructs, and also describe parameters for elastin expression from adult human SMCs in culture.

2. Results

2.1. Heparin treatment of cultured hAoSMC induces organized elastin fibers

As heparin has been shown to bind tropoelastin monomers *in vitro,* we hypothesized that heparin may affect the regulation of elastic fiber formation or organization *in vivo* (Tu and Weiss, 2008, 2010). To address this hypothesis, we treated adult human aortic smooth muscle cells (hAoSMC) with 300 μg/ml heparin for 7 days. To determine if heparin increased extracellular elastin protein level and organization, we stained these samples using immunofluorescence on nonpermeabilized cells (Fig. 1). Little elastin is observed in untreated samples (1A, 1B), however an increase in organized elastin fibers was found upon heparin treatment (Fig. 1C). The presented images use the Abcam antibody (see Methods), however nearly identical staining patterns were observed for all three antibodies tested. To confirm the identity of the fibers, we also stained these samples using a van Gieson-based elastin stain kit and observed an increase in extracellular elastin when treated with heparin (Fig. 1D) relative to untreated control (Fig. 1B).

2.2. Elastogenesis depends on initial cell density and heparin concentration

In these experiments, initial cell seeding density had a significant impact on elastogenesis. When cells were seeded at an initial density of 15,000 cells/cm^2, elastic fibers became visible by 3 days in culture, and became more numerous and interconnected by day 7 (Fig. 2). However, cells seeded at the lower initial density of 5000 cells/cm^2 developed far fewer and less extensive elastic fibers after 7 days in culture. Interestingly, cells seeded at the highest density tested, 30,000 cells/cm^2, also developed fewer and less extensive elastic fibers than those at 15,000 cells/cm^2. Despite the fact that each of the different seeding densities became confluent by 3 days in culture, the cell-cell interaction at the time of initial heparin treatment may

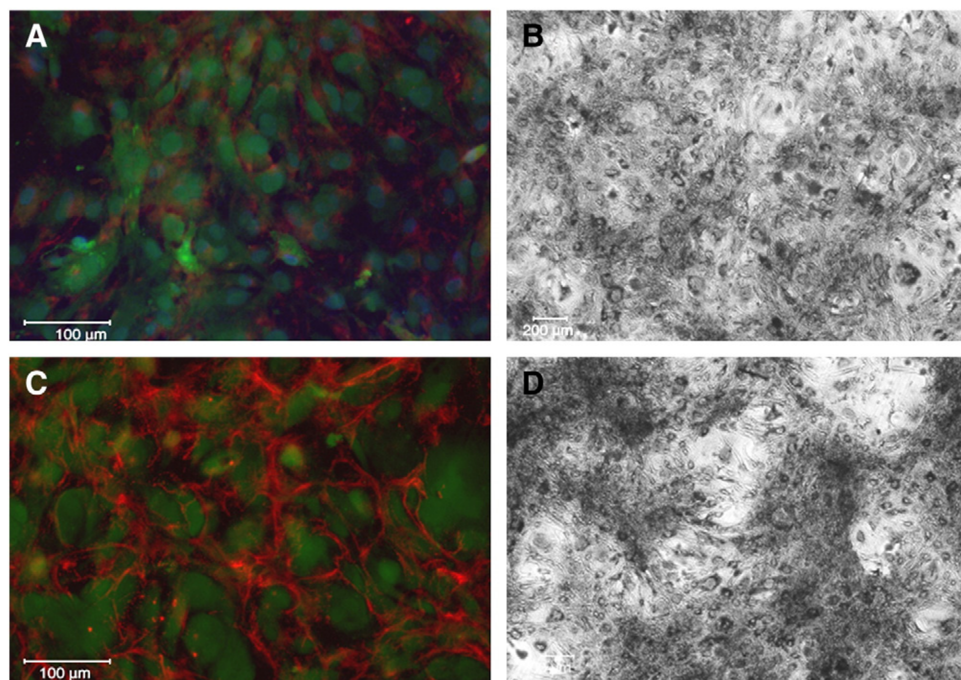

Fig. 1. Heparin treatment of primary hAoSMC shows organized elastin fibers. Adult human aortic SMCs were grown in the absence (A,B) or presence (C,D) of 300 μg/ml heparin for 7 days, then fixed and processed for immunofluorescence microscopy. Non-permeabilized samples were labeled with polyclonal anti-elastin antibody (red), CellTracker Green for cell membrane, and DAPI (blue) for nuclear stain (A,C); or with van Gieson stain for elastin (B,D). Significant extracellular elastin fibers are shown in the heparin treated samples.

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