Development of Naturally Derived Biomaterial Scaffold for Tissue Engineered Blood Vessel

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Statement of Purpose: The development of vascular prosthetic substitutes has evolved from entirely synthetic scaffolds to non-thrombogenic scaffolds, i.e. endothelialized scaffolds. Although the presence of endothelial cells (EC) has been shown to prolong the patency rates of implanted grafts in a variety of cardiovascular applications[1], the functional performance of these grafts still remains suboptimal compared to the native artery. One likely reason is the lack of vascular smooth muscle cells. Vascular smooth muscle cells (SMC), which mostly reside in the media layer, are attributed to the constriction and dilation of blood vessels. SMC play a vital role in permitting vascular segments to adapt to their mechanical environment. Moreover, SMC has been shown to benefit the endothelium by increasing EC retention and nitric oxide production[2], both essential in minimizing acute failure of implanted scaffolds. Taken together these attributes of SMC, the importance in seeding and impregnating scaffolds have become essential to enhance the contractility, mechanical stability, and long term patency of implanted grafts.

The goals of this project were to increase the attachment of SMC to decellularized scaffolds *in vitro*, and to accelerate the tissue-cell maturation/formation process. To accomplish these goals, a set of static seeding experiments were performed with decellularized porcine carotid arteries to determine the optimal cell seeding density, seeding environment, cell collection, and scaffold structural modification. Cyclic mechanical strain was followed within a bioreactor system to demonstrate the ability of the seeded SMC to proliferate, organize, align, and maintain functional characteristics on the scaffold. These experiments demonstrate the feasibility to seed vascular smooth muscle cells on decellularized scaffolds in order to develop functional scaffolds for the potential use in diseased arterial segments.

Methods: Primary cultured aorta smooth muscle cells were harvested from rats weighing approximately 600-700 grams. The adventitia was carefully removed and the remaining vessel was cut into approximately 1 mm^2 segments and incubated in culture medium into 6-well culture dish. Cells used for seeding experiments were between passages 2 through 5.

Carotid arterial segments were obtained from large pigs (400 to 600 lb, Nifong farm, NC). Vessels were washed in distilled water (D/W) and incubated decellularization solution (490 mL D/W, 10mL Triton-100X, 3.4 mL ammonium hydroxide) for 3 days in a mechanical rotating shaker at 4˚C. The decellularized vessels were then placed in washes, frozen for 24 hrs in an -80°C freezer, lyophilized (company), and sterilized with ethylene oxide.

To perform the static seeding, the decellularized scaffolds were first hydrated in the culture media and cut into 4mm x 4mm square segments. The square segments were then secured into individual wells of a 24-well culture dish. Careful consideration was taken to ensure the intima layers of the segments were pinned downwards. Twenty µL of SMC suspensions of 5 x 10^6 and 50 x 10^6 cells/mL were then pipetted onto each segment. H&E and DAPI staining were performed on each segment at 1 day, 3day, and 7 day time points.

In order to determine the effect of cyclic strain on SMC seeding, the decellularized segments were hydrated in the culture media for 3 hours and cut into rings of approximately 3 mm in width. The rings were placed on a custom made elastomeric silicone tube (Sylgard 184, Dow Corning Corporation, Midland, MI) with 4 mm outer diameter and a 0.5 mm wall thickness. The tube was positioned into a bioreactor were the rings could be submerged in culture media prior to static seeding. Twenty µL of BMSC suspension (50 x 10^6 cells/mL) was pipetted onto the top of each ring and incubated for 1 hour. This procedure was repeated as the sylgard tube was rotated 180 degrees. An additional 48 hours of static seeding was proceeded prior to positioning the bioreactor in a flow system. The flow system supplied a pulsatile flow waveform at a frequency of 1.0 Hz that induced a 5% strain in the sylgard tube. H&E and DAPI staining were performed to determine the proliferation and migration of the SMC onto the decellularized rings at 1 and 2 week time points.

Results/Discussion: In order to obtain collagen-rich rings, we decellularized segments of porcine common carotid arteries. These segments possessed an internal luminal size of 3-4 mm and were approximately 5 mm in length. Arterial segments that underwent decellularization and lyophilization maintained their tubular appearance and did not shrink significantly (Fig 1A). Hematoxylin and eosin (H&E) staining of decellularized vessels showed multiple layers of collagenous fibers within the vessel walls (Fig 1B). These results indicate that the decellularization process removes the native cells from the vessels, leaving a porous collagenous matrix.

To expose media layer to the SMC during seeding, the adventitia was removed from the decellularized porcine carotid arteries. The technical procedure successfully removed the adventitia, leaving the majority of the media layer intact (Fig 1C). The process could be easily repeated for any number of samples.

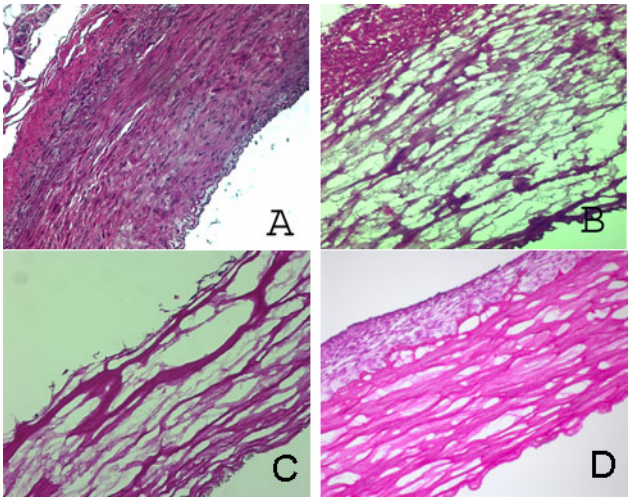

Figure 1. Decellularization of Porcine Arterial Segments. (A) Image of Native carotid artery. (B) H&E image of an decellularized porcine carotid. (C) De-adventitia decellularized porcine carotid artery. (D) 1 week dynamically conditioned decellularized scaffold.

The results from the static seeding experiments demonstrated that (1) an increase in cell density increased SMC attachment, (2) seeding the cells in a wet condition was more favorable then a moist environment, (3) an increase in cell attachment could be achieved with using trypsin to detach cultured SMC from tissue dishes as compared with scraping methods, and (4) the removal of the adventitia presented a more favorable matrix for cell attachment. The static seeding experiments were carried out to 7 days.

Mechanical strain was induced onto the seeded decellularized segments under physiological conditions with a pulse rate of 60 beats/min and a pressure range of 80 – 120 mmHg (figure 2) in a bioreactor system. Cross-sectional histological sections stained with Hematoxylin and Eosin was used to analyze cell proliferation, orientation, and re-alignment. The cells were seeded statically within the bioreactor for 48 hours prior to preconditioning. Preconditioning of the seeded scaffolds was implemented for durations lasting for 14 days. The results demonstrated that cells responded to the cyclic strain as early as 48 hours with re-orientation and re-alignment. Cell proliferation was continued through day 14 with an increase in realignment of cells (figure 3).

Conclusions: Developing a functional tissue engineered blood vessel remains a great challenge. To date, no vessel grown *in vitro* has been shown to display the contractile ability similar to native blood vessels. Some of these difficulties are likely related to long cell-tissue maturation periods *in* vitro which may have an adverse impact on cellular phenotype.

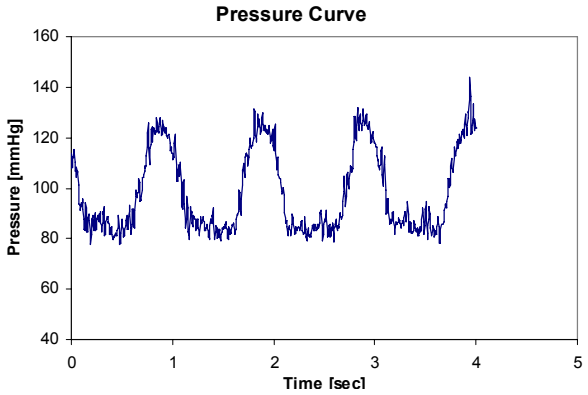

Figure 2. Pressure measurement of the bioreactor system

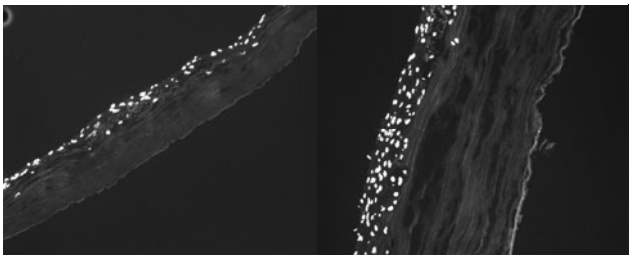

Figure 3. DAPI images demonstrating SMC proliferation and penetration with bioreactor preconditioning. (A) 7 day time of preconditioning decellularized rings with no adventitia seeded with SMC at a density of 50 million cells/mL. (B) 14 day time of preconditioning decellularized rings with no adventitia seeded with SMC at a density of 50 million cells/mL.

In this study, we demonstrate that SMC seeded on decellularized porcine carotid arteries adhere, proliferate, re-orient, and mobilize calcium under cyclic strain conditions. We have determined proper seeding methods and structural modification to ensure proper static seeding of SMC. Our findings of the advantageous of removing the adventitial layer in adhesion, proliferation, and calcium mobilization of SMC are promising, but it will be important to determine the specific proteins responsible for the adhesion and functional activity. In the future, the effects of serum concentration and strain amplitude/frequency on smooth muscle cell adhesion, proliferation, penetration, and function will be investigated in developing functional contractile tissue engineered blood vessels from decellularized arteries.

References:

1. Heyligers JM. Ann Vasc Surg 2005;19: 1-9.

2. Yu H. J Vas Surg 2003; 38: 557-563.