A SYSTEM TO CULTURE ENDOTHELIAL CELLS ON SMOOTH MUSCLE CELLS

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INTRODUCTION

Tissue engineering represents a promising approach to treat a number of cardiovascular problems including atherosclerosis, damaged valves and heart failure. Cultured blood vessels can be made from extracellular matrix alone or with smooth muscle cells embedded in polymer or collagen gel. Development of a functional, adherent endothelium is one of the major factors limiting the successful development of tissue-engineered grafts. Endothelial cells (EC) attachment and function on cultured blood vessels is limited and the adherent endothelium function in a procoagulant manner.

EC interactions with smooth muscle cells (SMC) influence vessel relaxation and contraction, SMC migration, and leukocyte adhesion. Communication occurs by diffusion and convection of soluble mediators such as nitric oxide (NO) and H_2O_2 and the movement of small solutes between cells through gap junctions. Developing cultured vessels that incorporate EC-SMC interactions is critical to creating functional vessels. Therefore, part of our work is aimed to develop a working model of EC-SMC interactions to elucidate the mechanisms and optimize the conditions by in which a functional endothelium may be incorporated into tissue engineered blood vessel.

In vivo and in vitro experiments show that EC influence the contractile and growth properties of vascular SMC [1]. SMC migration and growth is stimulated following removal of endothelium from the blood vessel. In culture, EC synthesize an extracellular matrix that inhibits smooth muscle cell proliferation that appears to be due to collagen and a heparin-like growth inhibitor [2]. Vasodilator expression is increased by application of fluid shear stress to cultured cells whereas vasoconstrictor release is increased by low shear stresses. EC derived NO inhibits SMC growth and migration and regulates vessel wall remodeling.

Generally, existing coculture systems bring EC and SMC within 10-50 μm to facilitate interactions. While these models have helped to elucidate many important interactions between the two cell types, a new system is needed in order to better replicate the in vivo spatial arrangement and apply to tissue engineered vessels. In order to test hypotheses about the effects of coculture and mechanical stimulation on EC and SMC function, we have begun to develop a coculture system in which both cell types are in direct contact. While reports of such a system exist in the literature [3], there has been no effort to optimize the culture conditions or assess the applicability of endothelialization of implanted tissue-engineered vessels.

METHODS

Porcine SMC and EC were initially cultured separately as described below. SMC and EC were harvested from a porcine proximal abdominal aorta [4]. All cells were used between passages 4 and 6. SMC and EC were cultured in proliferative medium (PM) [4] consisting of DMEM (Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS), 10% porcine serum (PS), 0.05 g L^{-1} vitamin C, 3□10^{-9} g L^{-1} $CuSO_4$, 0.05 M HEPES, 0.05 g L^{-1} proline, 0.05 g L^{-1} alanine, 0.02 g L^{-1} glycine, 10□10^{-9} g L^{-1} basic fibroblast growth factor, 10□10^{-9} g L^{-1} platelet-derived growth factor, and 1□ antibiotic/antimycotic (Sigma, St. Louis, MO).

Cocultures of SMC and EC (Figure 1) were prepared as follows. Proliferating subcultured SMC were plated at 20□10^3 cells cm^{-2} onto 10 □g cm^{-2} type I collagen (Sigma, St. Louis, MO) coated polystyrene SlideFlasks (Nalge Nunc International, Rochester, NY) in quiescent media (QM) consisting of DMEM/F12 media (1:1; Gibco Corp., Carlsbad, CA) supplemented with 1□ ITS (insulin, transferrin, and selenium) supplement and all non serum or growth factor PM supplements. This formulation was utilized previously to induce human SMC into a quiescent state and was successfully applied to porcine aortic SMC [5]. We also found that quiescent porcine aortic SMC exhibited increased expression of SM2 myosin and calponin compared to proliferating SMC, similar to the results obtained with human aortic SMC [5]. One day after plating SMC, a coating protein was added to the culture media - either 5 □g mL^{-1} type I collagen, 2.5 □g mL^{-1} fibronectin, or 1 □g mL^{-1} laminin (Sigma, St. Louis, MO). After allowing SMC to be coated overnight, proliferating EC were harvested and seeded at 40□10^3 directly onto the SMC/protein structure in QM supplemented with 1.5% FBS and 1.5% PS to ensure

EC attachment. Previous experiments had shown that without serum, EC would not attach effectively. The EC plating density was more than sufficient to cover the surface if only 50% of the cells attached. Cocultures were incubated for 2 or 5 days and then stained with 10 μg mL^{-1} AcLDL-DiI in PM to detect EC via their scavenger receptors. An epifluorescent microscope was then utilized to take pictures of the DiI fluorescence.

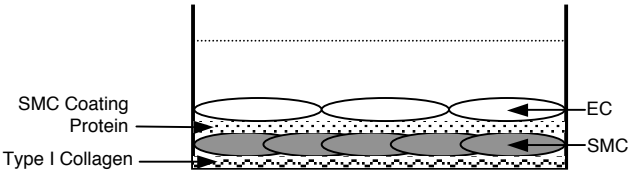

Figure 1. Organization of EC/SMC coculture

Analysis of the EC coverage was computed using the 50× fluorescent images of AcLDL-DiI uptake by EC. A Sobel edge detection filter using NIH Image 1.61 was first applied to he fluorescent images. The resulting images' background was then adjusted (compared to control EC group) and finally thresheld. Fine-tuning of the threshold level allowed pixel noise to be eliminated and calculation of EC coverage to be made. The data were then normalized to the EC control group, whereby 100% was the same coverage obtained in that group.

RESULTS AND DISCUSSION

Recent experiments show that quiescent SMC had 28±4% greater EC coverage than proliferative SMC (n=2). Also, it was seen in quiescent SMC cocultures that between 2 and 5 days after EC seeding, there was a decrease of 13±1% in apparent EC coverage (n=1).

Figure 2 shows a 200× epifluorescent image of a laminin coated EC/SMC coculture. This image was similar to the other groups in that good EC coverage was obtained. Greater AcLDL-DiI fluorescence was observed in coculture versus monolayer EC. This may be because EC are more active in coculture with additional uptake of AcLDL-DiI.

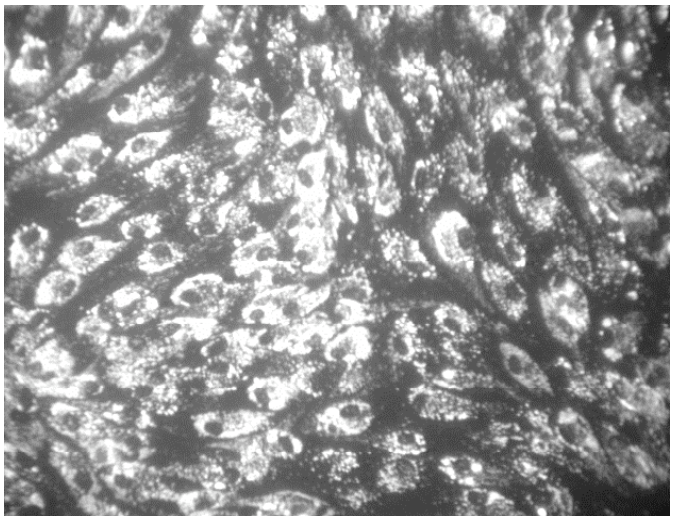

Figure 2. Epifluorescent image of SMC cocultured with EC (laminin as SMC coating protein) showing Ac-LDL DiI positive staining of EC (200×)

Shown in Figure 3 is the calculated coverage of EC after 2 days attachment on SMC with various coating proteins. Without any protein, EC coverage on quiescent SMC was 92±5%. However, in the optimal case with a laminin coating, the EC coverage increased to 106±10%. In the lowest coverage group, coating with type I collagen, coverage was still high, but not as consistent, 84±26%.

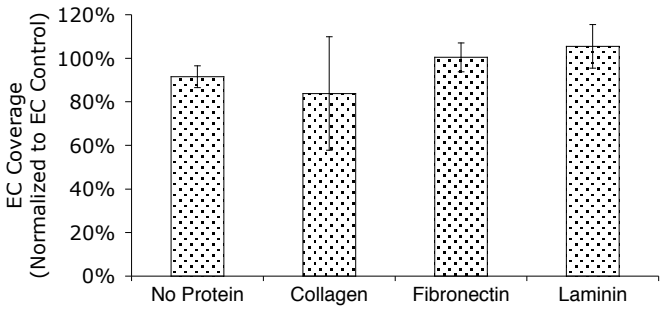

Figure 3. Change of EC coverage in coculture with various SMC coating proteins (n=2, 2 days post EC seeding)

The coating proteins appear to further enhance an already high coverage of EC on quiescent SMC. The optimal level that each is used at still needs examining, but the system is close to optimal under static conditions. Also, the protein by which the SMC are attached on the polystyrene is being examined to determine the optimal type and amount to use.

Current results are examining the coculture model under higher levels of scrutiny. Preliminary SEM results appear to confirm coverage and absence of SMC overgrowth. Once these results are confirmed, the functional level of the endothelial layer will be determined under flow conditions in a parallel plate flow chamber.

ACKNOWLEDGEMENT

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