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**Cellular growth under hydrostatic pressure  
using bovine aortic EC-SMC co-cultured ePTFE vascular graft\***

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**Abstract:** High blood pressure (hypertension) is implicated in the development of atherosclerosis. Blood vessels are constantly subjected to stretch due to blood pressure and changes in stretch usually instigate adaptive vascular remodeling, including abnormal growth and proliferation of vascular smooth muscle cells (VSMCs) as well as extracellular matrix (ECM). In this experiment, we used bovine aortic endothelial cells and smooth muscle cells (EC-SMC) co-cultured ePTFE vascular grafts subjected to normal atmospheric pressure (as a control), and 100 mmHg hydrostatic pressure for 7 d. The increase of cell layer thickness was observed. When measured, the cell layer thickness increased by 116.2%. The increase of collagen (Type IV) synthesis was also observed in the immunohistochemistry assay. When stained with toluidine blue, the cells showed metachromatic phenomenon.

**Key words:** Pressure, ePTFE graft, EC-SMC co-culture, Collagen, Proliferation, Metachromatic

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## INTRODUCTION

High blood pressure (hypertension) is implicated in the development of atherosclerosis. Blood vessels are constantly subjected to stretch due to blood pressure and changes in stretch usually instigate adaptive alterations of vessel wall shape and composition, which is called vascular remodeling. The abnormal growth and proliferation of vascular smooth muscle cells (VSMC) as well as extracellular matrix (ECM) are considered to affect the process of vascular remodeling greatly (Lehoux and Tedgui, 1998; Willis *et al.*, 2004), and such vascular remodeling is supposed to be related with pathogenesis of atherosclerosis.

It was shown that high hydrostatic pressure imposed on human aortic smooth muscle cells in culture affected DNA synthesis (Ozaki *et al.*, 1999) and cell proliferation (Iizuka *et al.*, 2001; 2004), and

reported that high hydrostatic pressure stimulated collagen synthesis of porcine aortic valve leaflets (Xing *et al.*, 2004).

Our present study is at testing the effect of hydrostatic pressure on the growth of the cell layer of a hybrid vascular graft constructed by co-culturing bovine aortic endothelial cells (ECs) and smooth muscle cells (SMCs) on an ePTFE vascular graft. We prepared a model of an arterial wall by seeding bovine aortic SMCs and ECs onto the luminal surface of ePTFE graft and co-culturing them, and then studied for the first time the effects of hydrostatic pressure on biosynthesis of collagen by the cells and their proliferation by measuring the thickness of the cell layer.

## MATERIALS AND METHODS

Bovine aortic smooth muscle cells (SMCs) were released from the culture dishes with trypsin containing 200  $\mu\text{g/ml}$  EDTA by volume. After centrifugation, the cells were suspended in Iscove's modified Dulbecco's medium (IMDM) containing

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Dulbecco's medium (IMDM) containing 20% fetal calf serum (FCS) by volume, 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml ascorbic acid. The SMCs were seeded onto the luminal surface of a 3.0-mm i.d., 4.0-cm long ePTFE graft previously treated with 70% ethanol and pre-coated with a layer of fibronectin, at a cell density of  $2 \times 10^6$  cells/cm<sup>2</sup> by means of pressure infusion of SMC suspension using a syringe, and they were cultured for 7 d in medium in a 95% air and 5% CO<sub>2</sub> humidified atmosphere at 37 °C. Then the ECs were seeded directly on the SMCs in the same manner, and they were co-cultured for 5 d until the ECs became confluent and completely covered the SMCs, forming a hybrid vascular graft. Then the graft was cut into two pieces: one of them was exposed to pressure of 100 mmHg, and another was placed under normal atmospheric pressure used as a control. They were all cultured for 7 d in a 95% air and 5% CO<sub>2</sub> humidified atmosphere at 37 °C. After 7 d culture, each piece of the graft was harvested, washed with PBS, then stained with hematoxylin-eosin (H&E) and toluidine blue. They were then subjected to immunohistochemistry assay. The stained histological specimens were observed under microscope and photographed, and the thickness of the cell layer was calculated by measuring and averaging the thickness at six different locations along the inner circumference of the graft.

## RESULTS

### **Moderately high hydrostatic pressure resulted in remarkable increase in the thickness of the cell layers**

After H&E staining, we could observe that ECs formed a monolayer at the surface of the tissue, while the proliferation of SMCs showed a difference at various pressure levels in 7 d culture. Cells and extracellular matrix were also seen depositing in the pores of the ePTFE graft wall (Fig.1). When measured at six different locations along the inner circumference of the grafts, the mean average thickness of the cell layer increased by 116.2% (*T* test) at 100 mmHg (Fig.2) when compared with that of control.

### **Moderately high hydrostatic pressure resulted in increase of collagen synthesis**

Immunohistochemistry assay showed that moderately high hydrostatic pressure stimulated the synthesis of collagen Type IV (Fig.3). The fact that compared with control, there was more brown colored collagen entangled about the cells exposed to 100 mmHg meant an increase in the amount of collagen.

### **Metachromatic phenomenon**

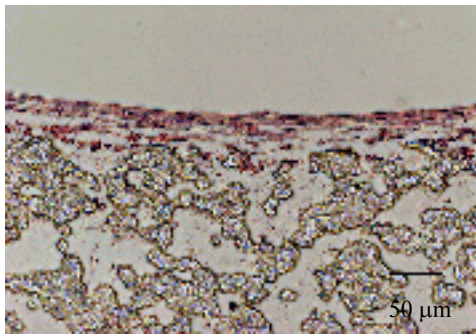
After being stained with toluidine blue, the cell layer on the graft exposed to 100 mmHg turned pink (metachromasia of toluidine blue), while the cells of control at normal atmospheric pressure turned blue (orthochromatic of toluidine blue) (Fig.4).

## DISCUSSION

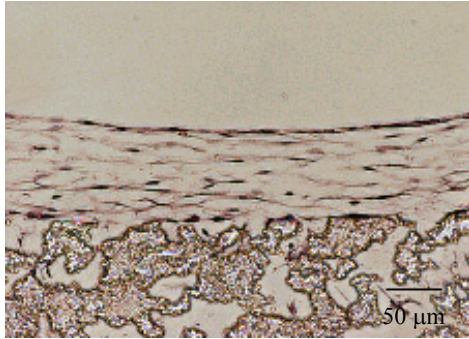
This study for the first time demonstrates that hydrostatic pressure can influence cellular growth and collagen synthesis of cultured hybrid grafts. This study provides a new method of cell co-culture by using the model of hybrid grafts, which is different from conventional cell culture in culture dishes. This study also yielded a new revelation that cells showed a particular character of metachromasia after being stained with toluidine blue.

Our results have several implications: (1) Moderately high hydrostatic pressure results in SMC proliferation on the luminal surface of hybrid grafts; (2) Moderately high hydrostatic pressure stimulates collagen synthesis of cells on the luminal surface of hybrid grafts; (3) The property of extracellular matrix secreted by cells would possibly change under high pressure.

In this experiment, we observed that after 7 d exposure to 100 mmHg, SMC proliferation and collagen synthesis increased when compared with control. We also found that after being stained with toluidine blue, the cells exposed to pressure showed a particular metachromatic character. There are two causative reasons for the result. First, it might be the influence of Phenol Red in the culture medium, since the pH of the medium for culturing the graft pieces at the pressure of 0 and 100 mmHg were 7.1 and 7.4 respectively. Second, and most importantly, it might be the effect of the changed secretion of the cells under high pressure, and it was likely to be some kind of proteoglycan. It is necessary to make further investigation.

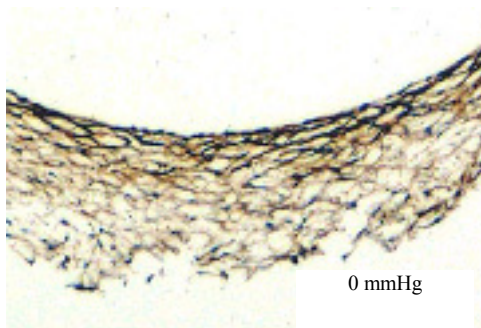


(a)

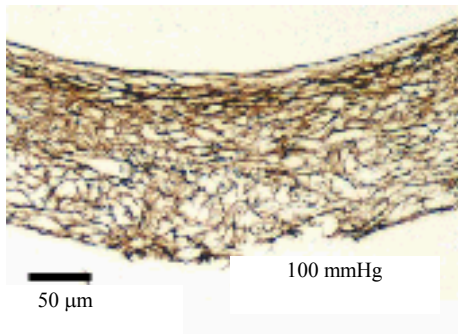


(b)

**Fig.1** The thickest parts of cell layers on individual graft pieces exposed to normal atmospheric pressure (as a control) (a) and 100 mmHg (b)

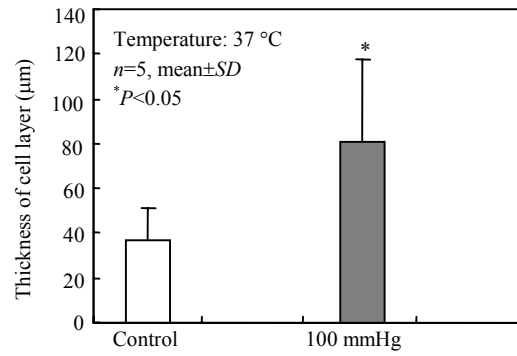


(a)

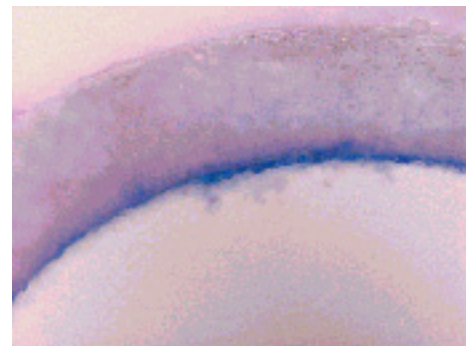


(b)

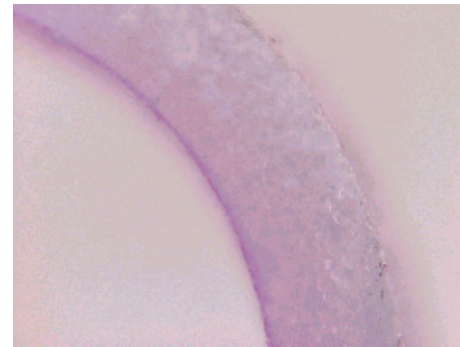
**Fig.3** Immunohistochemistry assay showed that compared with control (a), there was more brown colored collagen entangled about the cells exposed to 100 mmHg (b)



**Fig.2** Seven days exposure to 100 mmHg resulted in remarkable thickness increase (116.2%, *T* test) of cell layer when compared with that of control. Duration of incubation: 7 d



(a)



(b)

**Fig.4** After being stained with toluidine blue, the cells at normal atmospheric pressure turned blue (a), while the cells at 100 mmHg turned pink (b), showing the metachromatic character

From these studies, we can draw a conclusion that high hydrostatic pressure may cause SMC proliferation and increase of collagen synthesis in vitro, but the mechanism that governs it remains unknown. Also, vascular cells are subjected to complicated pulsatile pressure in vivo, and it was reported that flow shear stress also affected the growth of EC-SMC

co-culturing tissues in vitro (Niwa *et al.*, 2004). It is important to take those factors into consideration in future experiments.

In recent years, ePTFE vascular grafts are widely used in experimental and clinical studies (Williams *et al.*, 1994). It would be useful to investigate the situation of cellular growth on ePTFE grafts in vitro, and this experiment is the first that used EC-SMC co-cultured ePTFE grafts subjected to high hydrostatic pressure. It would be an essential part of tissue engineering and might be helpful to clinical application in vivo.

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