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Co-culture of endothelial cells and patterned smooth muscle cells on titanium: Construction with high density of endothelial cells and low density of smooth muscle cells

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ABSTRACT

Endothelialization has been considered a promising method to improve the biocompatibility of vascular implanted biomaterials. However, little is known about the anti-coagulation, anti-inflammatory, anti-atherosclerosis and anti-shedding property of the attached endothelial cells (ECs) and the relationship with their bio-environment and material-environment, which are both important evaluations to the cardiovascular biomaterials designed for tissue engineering applications and *in vivo* implantation. In this *in vitro* study, a novel co-culture model was built, where vascular smooth muscle cells (SMCs) were cultured on the hyaluronic acid (HA) micro-strip patterned titanium (Ti) surface on a low density to biomimetic the EC pericyte environment. Subsequently, the EC number and its functional factor, including nitric oxide (NO), prostacyclin (PGI_2), tissue factor pathway inhibitor (TFPI), thrombomodulin (TM), and the inflammatory induced factor, endothelial leukocyte adhesion molecule-1 (E-selectin) were quantified, respectively. The anti-shedding property was also assessed by the blood flow shear stress (BFSS) acting. The results showed that the novel co-culture model possessed better EC coverage, functional factor release and anti-shedding functions than the control.

1. Introduction

Cardiovascular diseases (CVDs) are not only the first cause of death worldwide, but also the leading cause of death in modern society [1]. According to World Health Organization (WHO) statistical data, there are about 17 millions of people who die from CVDs each year on a global scale [2]. After more than 30 years of development, percutaneous coronary intervention (PCI) has gradually matured and become a main treatment of CVDs. A lot of cardiovascular implants have been developed for the therapeutic of CVDs, including vascular grafts, stents, and artificial heart valves, et al. Nevertheless, a remarkable number of patients (up to 20%-30%) develop restenosis at the 3rd to 6th month after the PCI operation [3]. Most research attribute neointima formation and in stent restenosis to mechanical injury caused during the operation and the subsequent endothelial cell dysfunction, as well as thrombosis at the site of injury and smooth muscle cell (SMC) proliferation and migration [3]. In fact, the problem should be attributed to the inadequacy of the cardiovascular implant function on long-term antithrombotic and anti-hyperplasia which is relevant to their own biocompatibility [4]. Recent research generally consider that a confluent layer of endothelial cells (ECs) is the best surface to prevent adverse cardiac events [5]. Therefore, surface endothelialization has become the research hot tip of biomedical device surface modification and attracts more and more attention.

As mentioned above, surface endothelialization is an effective methodology for improving the biocompatibility of cardiovascular implants. Thus, biomaterials for improving their endothelial cell coverage are receiving increasing attention [6,7]. However, it is well known that endothelial cell function is influenced by complex vascular microenvironments, including extracellular matrix [8], pericytes [9], blood flow shear stress [10], etc. Smooth muscle cells (SMCs) as an important pericyte of endothelial cell, exist in two main types of cell forms: one type distributes around the vessel wall in contractile phenotype, constructing the middle membrane of the vascular [11]; the other type marginally distributes in the extracellular matrix between ECs and SMCs, and orients along the blood flow shear stress [12]. For decades, Several co-culture models have been developed to mimic the pericyte environment of ECs [12]. However, SMCs of these co-culture models have not been cultured in a bionic environment. So, building a biomimetic

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pericyte environment of ECs on the vascular implants' surface in the purpose of enhancing their physiological functions may be a better method.

In the present study, the ECs were co-cultured with low density micro-patterned SMCs on titanium (Ti). The anti-coagulation and anti-hyperplasia factor (NO, PGI_2, TM and TFPI) and inflammation relevant factor (E-selectin) of the attached endothelial cells were determined and evaluated, respectively. Additionally, the cell anti-shedding ability, as an important function, after exposed to the blood flow shear stress (BFSS) was also investigated.

2. Materials and methods

2.1. Micro-patterned surfaces

MP silicon templates with 25 μm wide lanes and 25 μm spacing were fabricated previously. Poly-dimethylsiloxane (PDMS) stamps were made from silicon templates by mixing at a 10:1 wt/wt ratio the 184-elastomer base to curing agent Sylgard, Dow Corning), and polymerizing at 120 °C. PDMS stamps were inverted on Ti substrates which were treated by 1 M NaOH solution at 80 °C for 24 h (samples labeled as TiOH), thereby creating micro-strips (samples labeled as TiOH/HAP), which were incubated for 24 h with 5 mg/ml hyaluronan (1×10^6 Da, Sigma). For non-patterned surfaces, TiOH substrates were used as control. The whole fabrication process is displayed in Fig. 1. The surface morphology of the TiOH and TiOH/HAP samples was characterized by Alcian-Blue-staining method and observed under a light microscope [13].

2.2. Smooth muscle cell and endothelial cell isolation

Primary culture of human umbilical artery smooth muscle cells (HUASMCs) and Human umbilical vein endothelial cells (HUVECs) were created by a conventional method which was described in the previous work [14,15], and the 3rd generation of HUASMCs and HUVECs were used in the experiment.

2.3. HUASMC density optimization for HUASMCs/HUVEC co-culture

HUASMCs in 5 different densities, 1×10^5 cells/ml, 5×10^4 cells/ml, 2.5×10^4 cells/ml, 1.25×10^4 cells/ml and 0.625×10^4 cells/ml, were seeded on the TiOH/HAP samples respectively, and cultured for 4 h, 1 day and 3 days each. A cck-8 assay was used to investigate the HUASMC proliferation. The medium was removed and the samples were washed three times with PBS. Then, fresh medium (have no phenol red) containing CCK-8 reagent was added to each sample and incubated at 37 °C for 4 h in standard culture conditions. Afterward, 170 μl of the blue solutions were transferred to a 96-well plate. The absorbance was measured at 570 nm by a micro-plate reader (BIO-TEK Instruments, USA). All proliferation experiments were performed in triplicate, and the seeded density of HUASMCs which caused a stable proliferation was chosen for HUASMC/HUVEC co-culture.

2.4. Co-culture of patterned HUASMCs and HUVECs

Cell Trackers with two different colors (red and green) were used in the whole co-culture process to distinguish the co-cultured HUASMCs and HUVECs clearly. The Tracker with red color was used for HUASMCs while the Green one was used for HUVECs, and the cells were firstly marked by the trackers before seeded. Briefly, HUASMCs were seeded on the patterned substrates at the density chosen in Section 2.3 and incubated at 37 °C for 4 h. After removing the culture medium and a tightly rinsed step with warm PBS (Sterile, pH 7.2), the micro-patterned HUASMCs were incubated with 50 μg/ml collagen IV (Sigma) for 1 h. Thereafter, HUVECs were plated onto the collagen IV layer at the density of 1×10^5 cells/ml and incubated at 37 °C for 1 day and 3 days, respectively. After rinsing three times with PBS in 37 °C, the cells were observed by fluorescence microscope. HUVEC number from 25 random optical microscope fields was counted and calculated statistically [16].

2.5. NO, PGI_2, thrombomodulin and tissue factor pathway inhibitor release of HUVECs

The amount of NO, PGI_2, thrombomodulin (TM) and tissue factor pathway inhibitor (TFPI) released from HUVECs on each sample were detected to evaluate the anticoagulation and inhibitory atherosclerosis properties of the HUVECs preliminarily. The NO, PGI_2, TM and TFPI release was examined by the methods as described in the previous work [17]. The amount of NO, PGI_2, TM and TFPI were finally normalized to cell number.

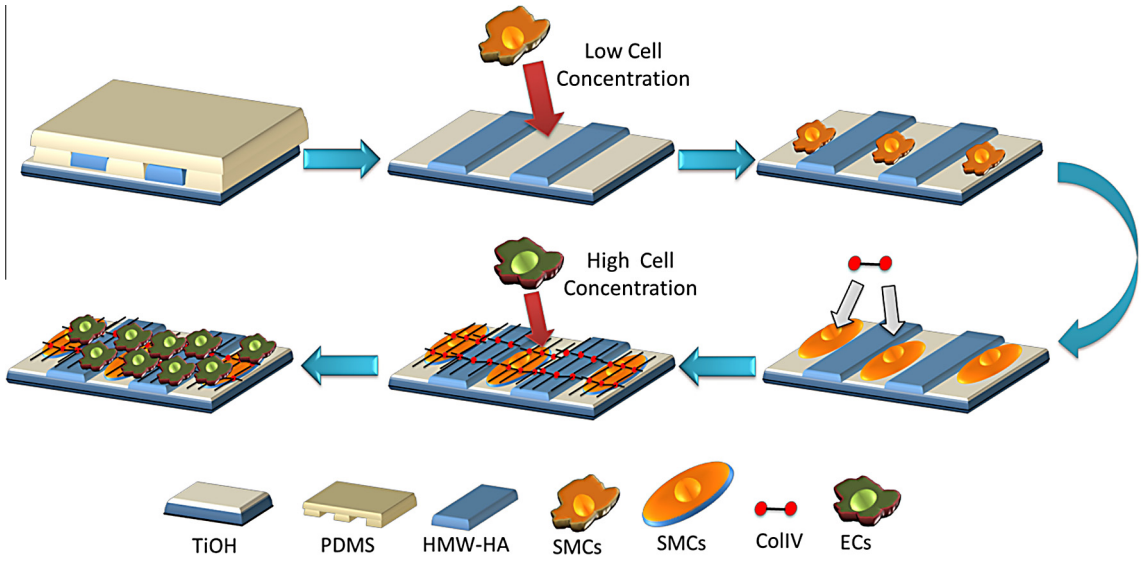

Fig. 1. The scheme of co-culturing endothelial cells and smooth muscle cells on TiOH/HAP substrate.