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Co-culture of vascular endothelial cells and smooth muscle cells by hyaluronic acid micro-pattern on titanium surface

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A B S T R A C T

Micro-patterning as an effective bio-modification technique is increasingly used in the development of biomaterials with superior mechanical and biological properties. However, as of now, little is known about the simultaneous regulation of endothelial cells (EC) and smooth muscle cells (SMC) by cardiovascular implants.

In this study, a co-culture system of EC and SMC was built on titanium surface by the high molecular weight hyaluronic acid (HMW-HA) micro-pattern. Firstly, the micro-pattern sample with a geometry of 25 μm wide HMW-HA ridges, and 25 μm alkali-activated Ti grooves was prepared by microtransfer molding (μTM) for regulating SMC morphology. Secondly, hyaluronidase was used to decompose high molecular weight hyaluronic acid into low molecular weight hyaluronic acid which could promote EC adhesion. Finally, the morphology of the adherent EC was elongated by the SMC micro-pattern. The surface morphology of the patterned Ti was imaged by SEM. The existence of high molecular weight hyaluronic acid on the modified Ti surface was demonstrated by FTIR. The SMC micro-pattern and EC/SMC co-culture system were characterized by immunofluorescence microscopy. The nitric oxide release test and cell retention calculation were used to evaluate EC function on inhibiting hyperplasia and cell shedding, respectively.

The results indicate that EC in EC/SMC co-culture system displayed a higher NO release and cell retention compared with EC cultured alone. It can be suggested that the EC/SMC co-culture system possessed superiority to EC cultured alone in inhibiting hyperplasia and cell shedding at least in a short time of 24 h.

1. Introduction

Neointima hyperplasia and thrombosis are the main problems for cardiovascular implants [1,2]. It has been reported that biomaterials with in vitro endothelialization can effectively inhibit thrombosis and intimal hyperplasia [3,4] after implantation into the body. However, endothelial cells (EC) cultured in vitro do not exhibit the physiological function due to the lack of pericytes as would be under normal physiological conditions. EC and smooth muscle cells (SMC) are the major cellular components of the vessel wall [5]. EC regulate the vascular tonus, permeability, inflammation, thrombosis, and fibrinolysis through the expression and secretion of a series of molecules [6]. While SMC play an important role for EC in the maintenance of normal cell morphology and function [7], such as the anticoagulant properties of EC and the release of biofunctional factors for inhibiting hyperplasia [8]. Therefore, it may be a promising method to build a co-culture system of EC and SMC on biomaterials surface to regulate the cell behavior, and further to investigate in vitro interactions of EC and SMC.

Several co-culture systems have been developed to investigate the interactions between SMC and EC, including culture of SMC and EC on opposite sides of semipermeable membranes [9], culture of EC on collagen gels containing SMC [10], culture of EC directly on SMC [11] and culture of EC on media layer which is covered by SMC [12]. Co-culture of EC and SMC on opposite sides of a thin membrane stimulated SMC proliferation [13], but the effects on EC has not been reported. Culturing EC directly on SMC also changes EC from the normal polygonal morphology in vitro to an elongated shape [14], whereas EC presents disordered behavior because the SMC below is also disordered. The above mentioned studies are helpful for understanding the interaction of biomaterials-EC-SMC to some extent, but few of the studies have been performed on an ordered surface. As known to all, the distribution of EC and SMC in human vascular wall is ordered and this distribution guarantees normal physiological functions of the two cell types. Thus, a co-culture system should be built on an ordered surface to study the

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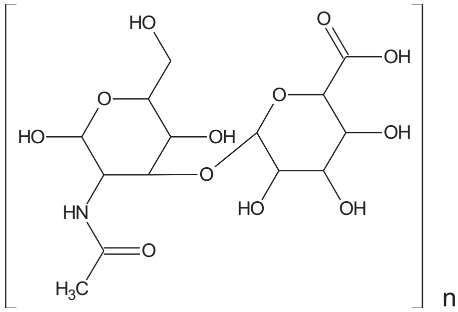

Fig. 1. Structure of hyaluronic acid.

interaction of biomaterials-EC-SMC under biomimetic conditions. The micro-pattern technology of biomolecule offers a promising approach to study the cell behavior on biomaterial surface [15]. Several kinds of biomolecule micro-pattern have been manufactured on biomaterial surface including groove/ridge stripes [16], square or round micro-domians [17], micro-networks [18], micro-tips [19], etc. Biomolecules such as heparin [20], fibronectin [21], collagen [22], silk fibroin [18] and arginine-glycine-aspartic acid (RGD) [23], etc. were used as single material or compounds to fabricate micro-pattern for promoting or inhibiting cell adhesion.

Here, hyaluronic acid (HA) has been chosen to fabricate a stripe micro-pattern, because HA from human tissue induces only weak immune reactions, additionally, the high molecular weight hyaluronic acid (HMW-HA) inhibits cell adhesion [24], while the low molecular weight hyaluronic acid (LMW-HA) promotes cell adhesion [25]. The key substance that makes the former into the latter is hyaluronidase (HAa). The chemical structure of HA [26] is shown in Fig. 1. It is reported that a parallel groove/ridge stripes micro-pattern can guide cell growth along the grooves [27], which are considered closest to physiological EC status exposed to blood flow shear stress [28]. Thus, HMW-HA micro-pattern can be fabricated to guide SMC growth along the grooves. Moreover, after HAa cleaves HMW-HA to LMW-HA, EC can adhere on the surface and grow on the ridge of the structure parallel to the SMC in the grooves. Thus the EC/SMC co-culture system can be applied to enhance the adhesion of EC and further promote the secretion of bioactive mediators by EC.

In the present work, we describe the construction, morphology, size and hydrophilicity of the HMW-HA micro-pattern on titanium (Ti) plates, and construct an EC/SMC co-culture system by the micro-pattern template for cardiovascular implants. As a preliminary study of co-culturing these two cells, the focus of functional assessment here is on EC nitric oxide (NO) release and anti-shedding that describes the stability of EC adhesion to the surfaces, which guarantee vascular endothelium anti-hyperplasia function and structure integrity [29]. Ti plates are chosen as the substrates because Ti and its alloys are the most widely used biomaterials for cardiovascular implants [30]. The modification of Ti substrate is characterized by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR) and water contact angle. To access the adhesion and anticoagulant function of EC in co-culture systems, the shear stress offered by a flow chamber is used and NO release amount is measured.

2. Materials and methods

2.1. Materials

HMW-HA (Sangon Biological Engineering Co. Ltd., China) and HAa (Sigma–Aldrich) were diluted to a concentration of 5 mg/ml and 10 μg/ml with phosphate buffered saline (PBS) solution at pH 7.2, respectively. Fibronectin (Fn, Sigma–Aldrich) was diluted to a concentration of 100 μg/ml with PBS at pH 7.2. Cell Tracker™ Orange CMTMR and Green CMFDA (Invitrogen, USA) were diluted to a concentration of 1 mmol/ml with sterile dimethyl sulfoxide, respectively. Medium F12, medium 199 (M199), fetal calf serum and type II collagenase were purchased from Gibco BRL. EC growth factor, trypsinization, Griess reagent, rabbit antihuman actin antibody, goat anti-rabbit IgG antibody and dimethyl sulfoxide were purchased from Sigma–Aldrich. All the other reagents were analytical of grade and used as received.

2.2. Methods

2.2.1. The preparation of HMW-HA micro-pattern on TiOH surface

The fabrication strategy of the model micro-patterned surface consists of three stages. The first stage was to fabricate the polydimethylsiloxane (PDMS) stamp. PDMS prepolymer was poured on a silicon surface with stripe micro-pattern (width of ridge 25 μm, width of groove 25 μm, 25 μm/25 μm), and then the PDMS stamp was peeled off from the silicon master after vacuum drying at 80 °C for 2 h. The PDMS stamp got a complementary relief structure against the silicon master. Secondly, 99.5% pure Ti plates (Baoji, China) were cut into round foils whose diameters were 10 mm and polished, then cleaned ultrasonically three times with acetone, ethanol, deionized water, and dried at room temperature. The cleaned Ti plates were soaked in a 1 M NaOH solution at 80 °C for 24 h, then rinsed thoroughly with deionized water (sample labeled as TiOH). Subsequently, 2 μl HMW-HA solution was added onto each TiOH surface (sample labeled as TiOH/HA), and subsequently the PDMS stamps were pressed down on the TiOH/HA surfaces by the force of 8 Newton for 12 h. Finally, micro-pattern on silicon master was moved to the TiOH/HA surface (sample labeled as TiOH/HAP) by PDMS stamp. All the preparation process is shown in Fig. 2.

2.2.2. Surface characterization

The morphologies of bare Ti, TiOH and TiOH/HAP samples were observed by scanning electron microscopy (SEM, JSM-7001F, Japan), and the surface chemical composition was examined using Fourier transform infrared spectrometry (FTIR, NICOLET 5700, USA) with reflectance mode. The wettability of the surface was assessed

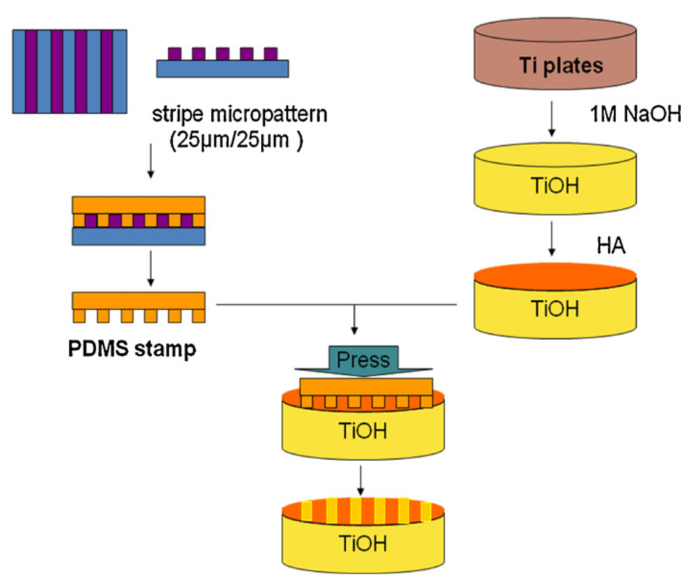

Fig. 2. Sketch map of the preparation of hyaluronic acid micro-pattern on TiOH surfaces.