Atherosclerosis 233 (2014) 590–600

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Three-dimensional multilayers of smooth muscle cells as a new experimental model for vascular elastic fiber formation studies

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ARTICLE INFO

Article history:
Received 9 August 2013
Received in revised form
20 January 2014
Accepted 21 January 2014
Available online 29 January 2014

Keywords:
Blood vessels
Smooth muscle cells
Elastic fibers
Nanotechnology
Tissue engineering
Fibronectin

ABSTRACT

Objective: Elastic fiber formation is disrupted with age and by health conditions including aneurysms and atherosclerosis. Despite considerable progress in the understanding of elastogenesis using the planar culture system and genetically modified animals, it remains difficult to restore elastic fibers in diseased vessels. To further study the molecular mechanisms, in vitro three-dimensional vascular constructs need to be established. We previously fabricated vascular smooth muscle cells (SMCs) into three-dimensional cellular multilayers (3DCMs) using a hierarchical cell manipulation technique, in which cells were coated with fibronectin-gelatin nanofilms to provide adhesive nano-scaffolds. Since fibronectin is known to assemble and activate elastic fiber-related molecules, we further optimized culture conditions.

Methods and results: Elastica stain, immunofluorescence, and electron microscopic analysis demonstrated that 3DCMs, which consisted of seven layers of neonatal rat aortic SMCs cultured in 1% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium, exhibited layered elastic fibers within seven days of being in a static culture condition. In contrast, the application of adult SMCs, 10% FBS, ε-poly(-lysine) as an alternative adhesive for fibronectin, or four-layered SMCs, failed to generate layered elastic fiber formation. Radioimmunoassay using [^{3}H]valine further confirmed the greater amount of cross-linked elastic fibers in 3DCMs than in monolayered SMCs. Layered elastic fiber formation in 3DCMs was inhibited by the lysyl oxidase inhibitor β-aminopropionitrile, or prostaglandin E_2. Furthermore, infiltration of THP-1-derived macrophages decreased the surrounding elastic fiber formation in 3DCMs.

Conclusion: 3DCMs may offer a new experimental vascular model to explore pharmacological therapeutic strategies for disordered elastic fiber homeostasis.

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1. Introduction

Arterial walls have a highly organized layer structure that consists of various cells and extracellular matrix (ECM) components. In particular, the vascular media is composed of a dense population of concentrically organized smooth muscle cells (SMCs) and elastic fibers, and plays a pivotal role in maintaining sufficient blood pressure, even during variations in hemodynamic stress. In physiological conditions, SMCs synthesize elastin and other specific molecules, which are incorporated into elastic fibers and arranged into concentric rings of elastic lamellae around the arterial media [1]. In contrast, arterial compliance and distensibility are impaired in the presence of cardiovascular disease and risk factors such as aortic aneurysm, atherosclerosis, ischemic heart disease, aging, hypertension, cigarette smoking, and diabetes [2]. Hence, impaired elastic properties are associated with arterial dysfunction and pathophysiology [2,3].

Changes in arterial elastic properties are the result of alterations in the intrinsic structural properties of the artery, including the fracturing and thinning of elastic fibers. Current approaches to

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0021-9150/$ – see front matter © 2014 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.atherosclerosis.2014.01.045

examining the elastogenesis and degradation of elastic fibers rely heavily on the use of the planar culture of vascular SMCs and genetically modified animals. These approaches have been instrumental in numerous discoveries and have been modified to create very elegant experimental designs [1,4–7]. Currently, however, no pharmacological strategy to promote elastogenesis and prevent the degradation of elastic fiber formation is available, and the molecular mechanisms of the regulation of elastic fiber formation remain to be studied. The two-dimensional (2D) monolayer culture system is a useful method for isolating specific factors and their effects on specific cell types [5–7], but it lacks the native-like layered structure of elastic fibers. Therefore, changes in the spatial arrangement of elastic fibers induced by various stimuli and the infiltration of immune cells cannot be observed. In-vivo analysis, on the other hand, often fails to discriminate among the various and complex factors. In this context, in vitro reconstruction vessel models overcoming these limitations are considered potential platforms of vascular biology that can provide further insights into the spatio-temporal molecular mechanisms of elastic fiber formation.

We previously developed a novel three-dimensional (3D) cell construction method [8] and created 3D-layered blood vessel constructs consisting of human umbilical arterial SMCs and human umbilical vascular endothelial cells [9]. To develop the 3D-cellular multilayers (3DCMs), we fabricated nanometer-sized cell adhesives like ECM scaffolds onto the surface of a cell membrane, which enables another cellular layer to adhere to the coated cell surface. We employed a layer-by-layer (LbL) technique to fabricate fibronectin–gelatin nanofilms onto living cell membranes, because the LbL technique produces nanometer-sized polymer films with a controllable nanometer thickness through the alternate immersion into interactive polymer solutions. We found that approximately 6 nm of fibronectin-based nanofilms were suitable for developing stable adhesive scaffolds and for creating allogeneic or xenogeneic multiple cell layers.

In addition to the cell adhesion effect, fibronectin has been known to orchestrate the assembly of the ECM [10–15]. In particular, recent reports suggest that fibronectin fiber assembles pericellularly into fibrillin microfibrils that have a complex structural organization and are widespread in elastic tissues [10,16]. Furthermore, fibronectin binds to lysyl oxidase (LOX), a cross-linking enzyme for elastic fibers, and acts as a scaffold for enzymatically active 30 kDa LOX [14]. Using scanning electron microscopy and transmission electron microscopy, we observed that fibronectin formed extracellular fibrils in the abovementioned 3DCMs within 24 h cultures [9], suggesting that fibronectin-coated SMCs have the potential to produce elastic fiber assemblies. In this context, we aimed to create the first scaffold-free 3D cellular multilayers (3DCMs) that are specifically designed for investigating the spatial regulation of vascular elastic fibers by employing this LbL assembly technique. The present study demonstrates that the optimized culture conditions provided layered elastic fiber formation in the 3DCMs consisting of neonatal rat SMCs within seven days of static culture conditions. In the 3DCMs, it was observed in the vertical view that macrophage infiltration or prostaglandin E_2 (PGE_2) changed the spatial arrangement of elastic fibers.

2. Materials and methods

Expanded materials and methods are described in Supplemental data.

2.1. Animals

Neonate (day 1) and adult Wistar rats (4–5months old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). All animal studies were approved by the institutional animal care and use committees of Yokohama City University.

2.2. Cell culture

Vascular SMCs in primary culture were obtained from the aorta of rat neonates (day 1) as previously described [17–19]. Briefly, the minced tissues were digested with a collagenase-dispase enzyme mixture at 37 °C for 20 min. The cell suspensions were then centrifuged, and the medium was changed to a collagenase II enzyme mixture. After 12 min of incubation at 37 °C, cell suspensions were plated on 35 mm poly-L-lysine-coated dishes The growth medium contained Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA). Human adult aortic SMCs were obtained from Lonza (Walkersville, MD, USA). The confluent SMCs were used at passages 5–7. THP-1 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan) and were maintained in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% FBS. All cells were cultured in a moist tissue culture incubator at 37 °C in 5% CO_2-95% ambient mixed air.

2.3. Construction of 3DCMs

Construction of 3DCMs was performed as previously described [8]. Briefly, a cell disk LF (Sumitomo Bakelite, Tochigi, Japan) was rinsed with 50 mM Tris–HCl buffer solution (pH 7.4) for 15 min and coated with fibronectin (0.2 mg/ml) for 30 min at 37 °C. SMCs were cultured on the cell disk (11×10^4 cells/cm^2) and incubated for 12 h in 10% FBS/DMEM. The monolayered SMCs were then immersed alternatively in a solution of fibronectin (0.2 mg/ml) and gelatin (0.2 mg/ml). After nine steps of LbL assembly with fibronectin and gelatin, a second cell layer was seeded on the first cell layer (11×10^4 cells/cm^2) and incubated for 6–12 h at 37 °C. The cycles of LbL nanofilm assembly and subsequent cell seeding were repeated six times in four days to construct seven-layered 3DCMs. During the first four days, the medium was refreshed daily. Twelve hours after the seeding of the last layer, the culture media was changed to DMEM or DMEM/F-12 (Gibco, Carlsbad, CA) containing 1% FBS or 10% FBS. The 3DCMs were incubated for an additional 48 h and either fixed in buffered 10% formalin or harvested in TRIzol (Invitrogen, Carlsbad, CA). The time-course of the 3DCM experiments is shown in Supplemental Fig. 1A. Stimulation by β-aminoproprionitrile (BAPN) or PGE_2 (1 μM) was performed simultaneously with the medium change on day 5. For macrophage infiltration assay, seven-layered 3DCMs at day 5 were put on a 24-well plate and applied with 500 μl of RPMI 1640 medium containing THP-1 cells (2.0×10^5 cells) with or without phorbol 12-myristate 13-acetate (PMA, 0.1 μM). The 3DCMs were incubated for 1 h at 37 °C. Next, unattached THP-1 cells were washed out with PBS and 3DCMs were incubated in 1% FBS/DMEM for 72 h until fixation. Control monolayered neonatal rat aortic SMCs (2D-SMCs) were plated on day 4 in the same density as a single layer in 3DCMs (11×10^4 cells/cm^2), and were incubated for 48 h. Four-layered 3DCMs were constructed from day 3 following the same time-course. The proportional increase in the thickness of 3DCMs with the number of seeding events was shown in Supplemental Fig. 1B. To confirm the effect of fibronectin nanofilms, ε-poly(lysine) (0.2 mg/ml) was used as an alternative adhesive polymer.

2.4. Quantitative measurement of insoluble elastin

Newly synthesized insoluble elastin was measured as previously described [20,21]. Briefly, after the seven-layered 3DCMs and 2D-

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