Biomaterials 35 (2014) 4739–4748

Contents lists available at ScienceDirect

Biomaterials

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

Effects of angiogenic factors and 3D-microenvironments on vascularization within sandwich cultures

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ARTICLE INFO

Article history:
Received 27 November 2013
Accepted 31 January 2014
Available online 18 March 2014

Keywords:
Artificial tissue engineering
Angiogenesis
Cytokine
ECM (extracellular matrix)
Endothelial cell

ABSTRACT

The in vitro fabrication of vascularized tissue is a key challenge in tissue engineering, but little is known about the mechanisms of blood-capillary formation. Here we investigated the mechanisms of in vitro vascularization using precisely-controlled 3D-microenvironments constructed by a sandwich culture using the cell-accumulation technique. 3D-microenvironments controlled at the single layer level showed that sandwich culture between more than 3 fibroblast-layers induced tubule formation. Moreover, the secretion of angiogenic factors increased upon increasing the number of sandwiching layers, which induced highly dense tubular networks. We found that not only angiogenic factors, but also the 3D-microenvironments of the endothelial cells, especially apical side, played crucial roles in tubule formation in vitro. Based on this knowledge, the introduction of blood and lymph capillaries into mesenchymal stem cell (MSC) tissues was accomplished. These findings would be useful for the in vitro vascularization of various types of engineered organs and studies on angiogenesis.

1. Introduction

A current key challenge in tissue engineering is in vitro vascularization of an engineered tissue that can be employed for clinically-relevant therapies and as a drug testing model. Blood capillaries, which are composed of endothelial monolayer, pericytes and fibroblasts, maintain the metabolic activities and functions of organs through the transportation of nutrients and oxygen [1]. The introduction of these vasculatures into engineered tissues has advantages to avoid necrosis of the inner tissues and to enhance their functions through cellular signaling [2,3]. To develop functional blood capillaries, many attempts such as a 3-dimensional (3D) culture of endothelial cells in hydrogels composed of extracellular matrix (ECM) [4,5], the fixation of angiogenic factors into scaffolds [6–11], cell sheet engineering for transplantable tissues [12–14], and micro channel models with flow [15–17] have been reported. Although these systems are powerful methods to construct vascularized tissues, they need complicated devices and procedures for the vascularization, and the potential limitations to recreate both the structures and functions of living tissues in vitro still remain. This is obviously caused by a poor understanding of the mechanisms responsible for the formation of blood-capillary networks in vitro, and therefore the requirements for vascularization should be clarified, such as the microenvironments for cell culture, the species and stiffness of the surrounding tissue, and the effects of angiogenic factors secreted under hypoxia.

Recently, we developed the rapid construction of 3D-vascularized multilayered tissues by the formation of ECM nanofilms onto single surfaces using layer-by-layer assembly [18]. Less than 10 nm thickness of ECM films composed of fibronectin and gelatin (FN-G) allowed all cells to adhere to each other through interactions between the FN-G nanofilms and the cell membrane proteins to create various types of tissues such as blood vessel walls and livers [19–22]. Using this technique and a sandwich culture, highly dense and homogeneous endothelial tubular networks were formed in fibroblast tissues. We have confirmed that this blood-capillary model can work as a model to test the differentiation stages of cartilage-like tissue [23].

Here, we report the biochemical and physical effects that can induce in vitro vascularization in engineered 3D-microenvironments (Fig. 1a). Since the cell-accumulation technique is able to control the number of tissue layers, we could investigate the role of the 3D-microenvironment at the level of a single cell layer. We tested the effect of the fibroblast layer number, angiogenic factors

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http://dx.doi.org/10.1016/j.biomaterials.2014.01.079
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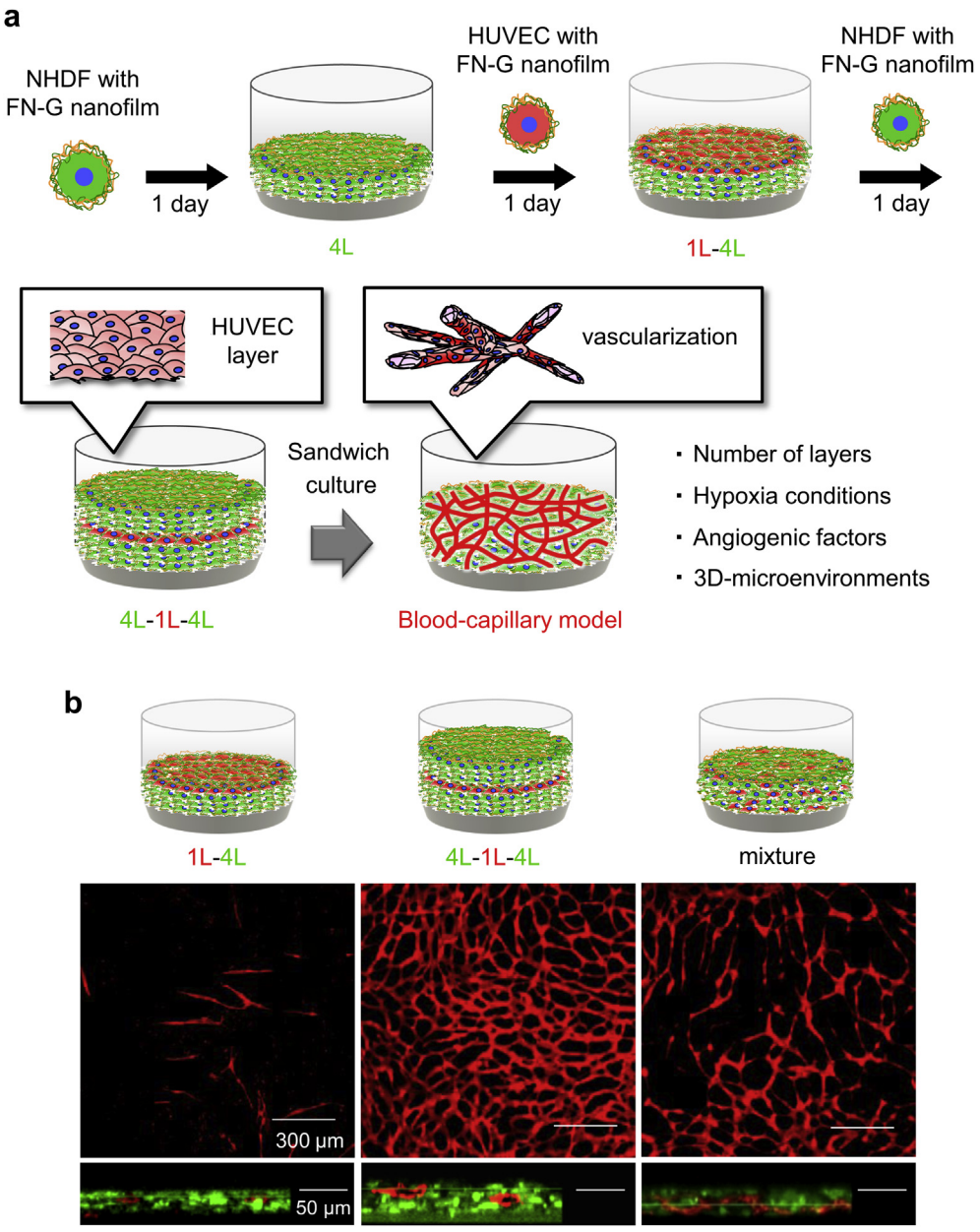

Fig. 1. In vitro vascularization by sandwich culture using the cell-accumulation technique. (a) Schematic illustration of the fabrication process of the endothelial tubule networks by the cell-accumulation technique. (b) CLSM cross-section images of 1L-4L tissue, 4L-1L-4L tissue, and a mixture of NHDFs and HUVECs after 7 days of incubation. The HUVECs were immunostained with an anti-CD31 antibody (red), and the NHDFs were labeled with CellTracker green (green).

secreted under hypoxic conditions, and 3D-microenvironments on the tubule formation of endothelial cells in vitro. Furthermore, the angiogenesis and lymphangiogenesis in human adipose-derived mesenchymal stem cell (MSC) tissues were also evaluated same as fibroblasts.

2. Materials & methods

2.1. Materials

All of the chemicals were used without further purification. Fibronectin (FN) from bovine plasma ($M_w = 4.6 \times 10^5$) and vascular endothelial growth factor (VEGF) were purchased from Sigma–Aldrich (MO, USA). Dulbecco's modified eagle medium (DMEM), gelatin (G) ($M_w = 1.0 \times 10^5$), tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), 10% formalin solution, and 4% paraformaldehyde (PFA)/phosphate buffer solution were purchased from Wako Pure Chemical Industries (Osaka, Japan). ε-Lys ($M_w = 4700$) was kindly donated by CHISSO Corporation (Chiba, Japan). The monoclonal mouse anti-human CD31 antibody and the monoclonal mouse anti-collagen type IV were purchased from Dako (Glostrup, Denmark). Goat anti-mouse Alexa Fluor 488- and 546-conjugated IgG, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), cell tracker green, Triton-X, fetal bovine serum (FBS), human adipose-derived mesenchymal stem cells (MSC), and MesenPRO RS medium were purchased from Life Technologies (CA, USA). The mouse anti-human VEGF antibody and the ELIZA assay kits for human VEGF, human hepatocyte growth factor (HGF) and human basic fibroblast growth factor (bFGF) were purchased from R&D systems (MN, USA). The cell culture insert with a 0.4 μm pore size was purchased from BD bioscience (NJ, USA) and Corning (NY, USA). Normal human dermal fibroblast (NHDF), human umbilical vein endothelial cell (HUVEC), human umbilical artery endothelial cell (HUAEC), human dermal lymphatic microvascular endothelial cell (LEC), and endothelial growth medium (EGM-2MV) were purchased from Lonza (NJ, USA). Mouse 10T1/2 and 3T3 cells were purchased from ATCC (VA, USA). The mouse pancreatic fibroblast cells (K643f) and mouse 3T3 fibroblast cells were kindly donated by Dr. M. R. Kano from Okayama University and Dr. K. Miyazono from Tokyo University.