THE EFFECT OF SHEAR STRESS ON MICRO-VESSEL NETWORK FORMATION OF ENDOTHELIAL CELLS

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INTRODUCTION

Angiogenesis plays an important role in vascular biology because it is an essential process that occurs in various pathological events such as arteriosclerosis, diabetes, and tumor growth.

Sprouting angiogenesis involves a growth phase and a stabilization phase of vascularized vessels. During the initial phase, a dissolution of the basement membrane of the existing vessel and its sorrounding extracelluar matrix is followed by migration and proliferation of endothelial cells in the created space, lumen formation within the endothelial sprout comes next, resulting in the formation of loops by anastomoses of sprouts. In the stabilization phase, endothelial cell proliferation is arrested and after reconstruction of a basement membrane around the neovascular, the immature capillary is covered with pericytes.

The concept that tumor development depends on angiogenesis has been established and several angiogenic molecules have been identified, including the fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) families [1]. The basic fibroblast growth factor (bFGF) in particular may play a pivotal role in vascular remodeling. Several investigations have reported that this cytokine has a critical vote in new vessel formation [2,3]. Although major progress has been made, our understanding of the molecular mechanisms of these processes is still incomplete.

In addition to angiogenesis, the existence of bone marrow-derived endothelial progenitor cells were characterized to be circulating in the blood of adult animals and their incorporation into pathological neovascular sites indicate that vasculogenesis may also participate in pathological neovascularization[4].

Some studies have demonstrated that function and morphology of the vascular systems are regulated by hemodynamic stress. Among the rheological components, wall shear stress is believed to play a considerable role in vascular remodeling. When blood flows over the endothelial wall, shear stress is loaded on the luminal surface endothelial cells of the blood vessel. *In vivo* studies suggested that wall shear stress result from a blood flow induced adaptive changes in large blood vessels diameter. It acts as a negative feedback to maintain the stress constant around its physiological level. *In vitro* studies using cultured endothelial cells have also revealed that application of fluid shear stress modifies various cell functions and morphology with alterations in gene expression. Thus wall shear stress is thought to a prime factor in regulating adaptive vessel growth. It is also believed that shear stress represents a significant stimulus for the formation of new vessels (angiogenesis) [3,5]. At the same time, little is known about the role of shear stress, about mechanosensing procedures or about potential mediators involved in shear stress response.

In vitro experiments, great advances in the understanding of angiogenesis have been provided by three-dimensional models. This assay is based on the capacity of stimulated endothelial cells to invade three-dimensional substrates. When confluent cells cultured on gels are activated by cytokines such as bFGF or by phorbol esters, they invade the underlying gel and form capillary-like structures [2]. Three-dimensional models are closer to an *in vivo* environment than two-dimensional ones (*e.g.* Matrigel model), because they consider more steps of angiogenesis. Depending on the culture media composition, cells can be induced to sprout, proliferate, migrate, or differentiate in the 3-D form [6].

To examine the effects of shear stress, we observed the changes of micro-vessel formation in a three-dimensional angiogenesis model due to shear stress loaded.

EXPERIMENTAL PROCEDURES

Cell Culture

Shear stress experiments were performed using bovine pulmonary microvessel endothelial cells (BPMEC, Cell Systems). The endothelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (JRH Biosciences) and 1% antibiotic-antimycotic (Gibco) and cultivated under standard conditions (37°C, 5% CO_2).

In Vitro Angiogenesis Assay

Collagen gels were prepared as following: 8 volumes of a solution of type I collagen (Nitta Gelatin) were quickly mixed with 1 volume of 5x DMEM and 1 volume of 0.1 N NaOH (Wako Pure Chemicals Industries) on ice. The gels were dispensed into a glass-base dish and allowed to gel at 37°C for 30 minutes.

BPMEC were seeded onto three-dimensional collagen gels at $4x10^5$ cells/35 mm culture dish. Subsequently, 30 ng/ml bFGF (Pepro Tech) were added after reaching confluence.

Application of Shear Stress

Three-dimensional angiogenesis models were transferred into a parallel-plate flow chamber, made of polycarbonate and designed to subject cells grown on collagen gels to well defined laminar fluid shear stress. Fluid flow was provided by a sterile continuous flow loop. The width of the parallel-plate flow chamber is much larger than its height. Therefore, the sidewalls have minimal impact on the shear stress distribution along the bottom surface. The flow volume is set up to apply a laminar shear stress of 0.5 Pa and then maintain this volume for 48 h at 37°C.

Quantification of Invasion

Fields in which BPMEC began to form three-dimensional networks were selected and photographed every 10 minutes by phase contrast microscopy. Invasion was quantified by determining the total additive length of cell cords.

RESULTS AND DISCUSSION

FGF Induces Angiogenesis *in Vitro*

To assess the effect of shear stress on micro-vessel formation, we have used an *in vitro* three-dimensional model in which endothelial cells can be induced to invade a collagen gel. BPMEC formed a confluent monolayer on the surface of the collagen gel 72 hours after seeding the cells. Subsequently, we added bFGF and the cells invaded the underlying gel to form an extensive network (Figure 1).

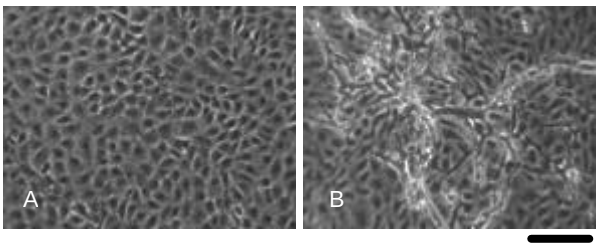

Figure 1. *In vitro* model of network formation
Endothelial cells grown to confluence on the surface of a three-dimensional collagen gel (*A*) were treated with bFGF (30 ng/ml) for 48 hours (*B*). Bar, 100 μm.

Exposure of Endothelial Cells to Shear Stress

After exposing 1 hour of laminar shear stress (0.5 Pa), the collagen gel underlying endothelial cells caved in and the shear stress applied to endothelial cells varied in 0.30 ± 0.03 Pa (n = 5). This was maintained throughout this experiment.

Although, in static cultures, the BPMEC on the surface of gels shows migration in random directions, the cells that had been subjected to a laminar shear stress migrated with the direction of flow after approximately 8 hours of shear stress. This phenomenon continued throughout the time of exposure to shear stress, but elongation and alignment of endothelial cells were not observed. We believe that the shear stress is probably too small to cause these events.

Effect of Shear Stress on Micro-Vessel Network Formation

After 24 hours of treatment with bFGF, cells were subjected to laminar shear stress or the static culture condition for 48 hours. In time course experiments, elevated levels of network formation were detectable after 12 hours of shear stress. Whereas, networks under static condition constantly developed (Figure 2). This demonstrates that shear stress is a potent stimulus in the growth of micro-vessel network formation. It was consistent with the established results that angiogenesis was up-regulated by shear stress *in vivo* [5] and shear stress developed two-dimensional capillary-like structure 24 hours after the flow was stopped *in vitro* [3]. This study revealed that three-dimensional network formation could be induced under shear stress subjected condition.

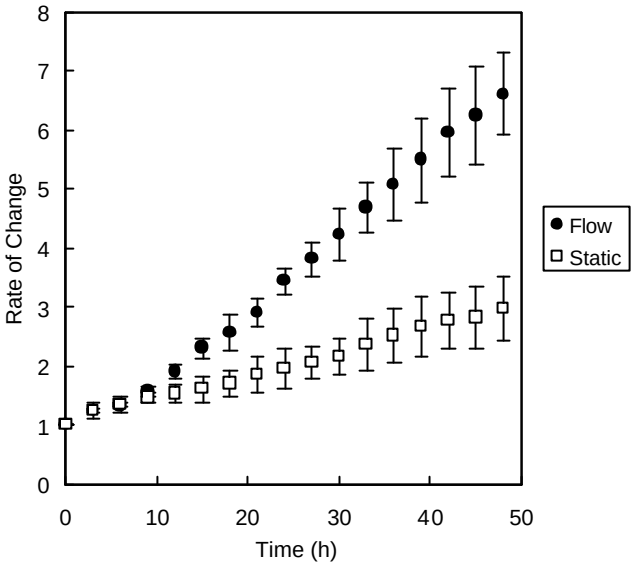

Figure 2. Time course change of network formation
Total length of micro-vessel network of either static cells or shear-exposed cells was calculated at every 3 hours.

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