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Bioactive silicate materials stimulate angiogenesis in fibroblast and endothelial cell co-culture system through paracrine effect

H. Li [a], J. Chang [a,b,*]

[a] Med-X Research Institute, School of Biomedical Engineering, Shanghai Jiao Tong University, 1954 Huashan Road, Shanghai 200030, China
[b] Shanghai Institute of Ceramics, Chinese Academy of Sciences, 1295 Dingxi Road, Shanghai 200050, China

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ABSTRACT

Angiogenesis is critical in tissue engineering, and bioceramic-induced angiogenesis has been reported. However, the role of other types of cells such as fibroblasts in this bioceramic-induced angiogenesis process has not been reported, and is closer to the in vivo situation of tissue regeneration. In this study, the paracrine effect of silicate bioceramic-induced angiogenesis in the presence of fibroblasts was confirmed by investigating the effects of calcium silicate (CS), one of the simplest silicate bioactive ceramics, on angiogenesis in co-cultures of human dermal fibroblasts (HDF) and human umbilical vein endothelial cells (HUVEC). Results showed that CS extracts stimulated the expression of vascular endothelial growth factor (VEGF) from co-cultured HDF and subsequently enhanced the expression of VEGF receptor 2 on co-cultured HUVEC (co-HUVEC). The endothelial nitric oxide synthase and nitric oxide production in co-HUVEC was then increased to finally initiate the proangiogenesis. During this process, the expression of vascular endothelial cadherin from co-HUVEC was up-regulated, and cadherin proteins were concentrated at the cell junctions to facilitate tube formation. Silicon ions are confirmed to play an important role during silicate bioceramic-inducing angiogenesis, and effective silicon ion concentrations (0.7–1.8 μg ml^{-1}) are proposed.

1. Introduction

Tissue engineering (TE) provides a promising way to regenerate damaged or lost tissue/organs by combining cells and biomaterial scaffolds to reconstruct and regenerate new tissue or organs. However, for the survival of a tissue-engineered construct with size larger than the diffusion limit of oxygen after its implantation in vivo, the tissue has to be vascularized, which means that a capillary network capable of delivering nutrients to the cells has to be formed within the construct [1]. Therefore, angiogenesis has recently been recognized as one of the key challenge in TE [2]. The speed of the spontaneous vascular ingrowth from the existing blood vessels to the tissue induced by in vivo signals is normally several tenths of micrometers per day, which is too slow to provide adequate nutrient to the cells in the interior of the construct [3,4]. Therefore, additional strategies for stimulating angiogenesis are essential to ensure the survival of large tissue-engineered constructs.

As angiogenic growth factors, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been widely used for stimulating angiogenesis in tissue-engineered constructs [5–7]. Unfortunately, delivery of growth factors may be limited in TE technology, as the proteins may be denatured during the processing of TE scaffolds or biomaterials, which usually involves conditions that are not favorable for proteins. In addition, the release behavior of proteins in vivo cannot be easily controlled, owing to the complicated in vivo environment and short half-life of proteins, which may result in possible risks [8].

Recently, biomaterial-based proangiogenesis was proposed, as it has been reported that some silicate bioactive materials possess proangiogenic potential, which may provide an alternative to the application of recombinant inductive growth factors [8–15]. A detailed review of studies investigating bioactive glasses with respect to angiogenesis by Gorustovich et al. [8] has been published recently. Experiments have shown that bioactive glasses stimulate the proliferation of endothelial cells and the formation of endothelial tubules [11,12,14]. Zhai et al. [15] reported that, in addition to bioactive glasses, a Ca–Mg–Si-containing bioceramic, akermanite, enhanced angiogenesis of human aortic endothelial cells, and it was proposed that silicon ions might play an important role in the stimulation of angiogenesis.

It has been reported that, in addition to endothelial cells, other cells, such as fibroblasts, are also involved in the angiogenic process [16–18]. Therefore, an in vitro cell co-culture model with fibroblasts and endothelial cells has been used to study the

* Corresponding author at: Shanghai Institute of Ceramics, Chinese Academy of Sciences, 1295 Dingxi Road, Shanghai 200050, China. Tel.: +86 21 52412804; fax: +86 21 52413903.
E-mail address: jchang@mail.sic.ac.cn (J. Chang).

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mechanisms of angiogenesis, and the results showed that co-culture of endothelial cells with fibroblasts could significantly enhance the angiogenesis of endothelial cells [16–18]. The importance of extracellular matrix formed by fibroblasts for the angiogenesis in the co-culture system has been reported [16,17]. In addition, Montesano et al. [18] reported that endothelial cells were induced to initiate angiogenesis by Swiss 3T3 fibroblasts through the paracrine effect, as the presence of fibroblasts could increase plasminogen activator activity in endothelial cells. These reports indicated that there are intimate communications between fibroblasts and endothelial cells for angiogenesis, and the paracrine effect may play an important role in these communications.

Many experiments have demonstrated that the bioactive glass also stimulates the secretion of angiogenic growth factors in fibroblasts when they were co-cultured with endothelial cells, which finally enhances the angiogenesis [11,12,14]. In indirect contact co-cultures of fibroblasts and endothelial cells, the bioactive glass dissolution products were found to be able to stimulate human colon fibroblasts to secrete bFGF and VEGF, and the conditioned media collected from these cells enhanced endothelial cell proliferation and tubule formation [11,14]. In direct contact co-culture of fibroblasts and endothelial cells, Leu and Leach [12] found that the tube formation was enhanced in the co-culture system. Although they did not study the expression of VEGF or bFGF in the co-cultured cells, they suggested that the potential contribution of fibroblast-secreted angiogenic factors stimulated by bioactive glass in tubule formation might be important [12]. However, it is still unclear how the endothelial cells respond to the angiogenic factors expressed by fibroblasts under the stimulation of biomaterials, since these studies did not further study the downstreaming biological responses of endothelial cells in the angiogenesis process.

Interestingly, a paracrine effect through angiogenic factors was also observed in the direct contact co-culture of HUVEC and human bone marrow stem cells (HBMSC) without biomaterials in previous studies [19–21]. It was found that the co-culture of HUVEC and HBMSC obviously enhanced the formation of capillary-like networks through paracrine effects in the following way: the co-cultures upregulated the expression of VEGF in HBMSC; VEGF then activated its receptor KDR and urokinase-type plasminogen activator in co-cultured HUVEC. Finally, the migration of endothelial cells was initiated and the self-assembled networks were formed [19].

Thus, it can be hypothesized that the way in which the silicate bioactive materials stimulate the angiogenesis of endothelial cells in the presence of fibroblasts might also be the paracrine way: the silicate bioactive materials stimulate fibroblasts to express angiogenic factors, which subsequently activates their receptors on endothelial cells and initiates downstreaming cascades; finally, the angiogenesis of endothelial cells is stimulated.

In addition to the angiogenic factors that stimulate angiogenesis, it has been widely recognized that formation of tubular-like network structures are critical steps for angiogenesis, and need the participation of adherens molecules [22,23]. In endothelial cells, the predominant adherens molecule is vascular endothelial cadherin (VE-cad), which is endothelial cell-specific and strictly located at the junctions of endothelial cells in blood vessels [24–26]. The role of VE-cad in determining the endothelial cell contact integrity, controlling the cellular junction and tubular-like network formation, has been extensively demonstrated through functional studies in mono-cultures of endothelial cells [24–26]. In addition, a previous study demonstrated that the VE-cad is critical for angiogenesis in the HBMSC and HUVEC direct contact co-culture system, as blocking the expression of VE-cad significantly suppressed the self-assembled capillary-like network formation [19]. The co-culture system stimulated the VE-cad expression and the over expressed VE-cad concentrated at the cell junction, acting like "glue" to glue endothelial cells together, and stimulated angiogenesis. Since VE-cad is a critical adherens molecule in the communication of endothelial cells and other types of cells, the effects of biomaterials on the VE-cad expression and distribution in endothelial cells co-cultured with fibroblasts also need to be studied, which is critical for understanding the mechanism through which the silicate bioactive materials stimulate the angiogenesis.

Therefore, the aims of this study are to confirm the hypothesis that the stimulation of angiogenesis of co-cultured endothelia cells and fibroblasts by silicate bioactive materials is through paracrine effects, and to identify the role of the adherens molecule VE-cad in this biomaterial-induced angiogenesis process. In previous studies of biomaterial-induced angiogenesis, silicate bioactive glasses [8–14] and akermanite [15] were investigated. These two types of material contain many different elements, such as Ca, Si, Na, P and Mg, among which Si and Ca are thought to play a more important role in the stimulation of angiogenesis than the other elements. In order to be able to identify the role of the critical components of silicate bioactive materials in the angiogenesis process, i.e., Ca and Si, calcium silicate (CS) was used in this study, as it is one of the simplest silicates only containing Si and Ca. A co-culture system of fibroblasts and endothelial cells was applied as the cell model, and both indirect and direct contact co-culture models were used to investigate the effects of CS ion dissolution products on the two types of cells in terms of the expressions of VEGF and its receptors, the downstreaming endothelial nitric oxide synthase (eNOS) and nitric oxide (NO).

2. Materials and methods

2.1. Materials

CS powders were prepared by a chemical coprecipitation method [27,28]. Briefly, continuous mixing of an aqueous solution of Na_2SiO_3 (1 mol l^{-1}) with an aqueous solution of $Ca(NO_3)_2$ (1 mol l^{-1}) at ambient temperature was carried out overnight (mol ratio: Na_2SiO_3:$Ca(NO_3)_2$ = 1:1). Then the stirring was stopped, and the resulting CS suspension was filtered and washed, first with deionized water and subsequently with ethanol. After being dried at 80 °C overnight and calcined at 800 °C for 2 h, the CS powders obtained were sieved to obtain particles between 100 and 150 μm for future use.

2.2. Ion extract preparation and ion concentration determination

The extracts of CS bioceramics were prepared according to the procedures reported in the literature [28–30]. Briefly, 1 g of bioceramic powder was soaked in 5 ml serum-free endothelial basal medium-2 (EBM-2, Lonza) and incubated in a humidified 37 °C/5% CO_2 incubator for 24 h. The supernatant was collected, sterilized through a filter (Millipore, 0.22 mm) and stored at 4 °C (ISO10993-1) for further use [29,31]. The calcium silicate extract is referred to as CS extract.

CS extracts were diluted with DMEM (GIBCO)+10% fetal bovine serum (FBS) at the ratios of 1/16, 1/32, 1/64, 1/128 and 1/256. To determine the ion concentration in bioceramic extracts, 5 ml diluted samples taken from the media for cell culture were checked in terms of Ca, Si and P ion concentrations by inductively coupled plasma atomic emission spectroscopy (ICP-AES; Optima 3000DV, Perkin Elmer, USA). DMEM+10% FBS was also analyzed by ICP-AES as control medium.

2.3. Cell isolation and culture

Human dermal fibroblasts (HDF) were isolated and cultured from the superficial layer of adult human skin dermatomed at a