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Silicate bioceramics enhanced vascularization and osteogenesis through stimulating interactions between endothelia cells and bone marrow stromal cells

Haiyan Li [a,1], Ke Xue [b,1], Ni Kong [a], Kai Liu [b,**], Jiang Chang [a,c,*]

[a] Med-X Research Institute, School of Biomedical Engineering, Shanghai Jiao Tong University, 1954 Huashan Road, Shanghai 200030, China
[b] Department of Plastic and Reconstructive Surgery, Shanghai 9th People's Hospital, Shanghai Jiao Tong University School of Medicine, 639 Zhizaoju Road, Shanghai 200011, China
[c] Shanghai Institute of Ceramics, Chinese Academy of Sciences, 1295 Dingxi Road, Shanghai 200050, China

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The facts that biomaterials affect the behavior of single type of cells have been widely accepted. However, the effects of biomaterials on cell–cell interactions have rarely been reported. Bone tissue engineering involves osteoblastic cells (OCs), endothelial cells (ECs) and the interactions between OCs and ECs. It has been reported that silicate biomaterials can stimulate osteogenic differentiation of OCs and vascularization of ECs. However, the effects of silicate biomaterials on the interactions between ECs and OCs during vascularization and osteogenesis have not been reported, which are critical for bone tissue regeneration *in vivo*. Therefore, this study aimed to investigate the effects of calcium silicate (CS) bioceramics on interactions between human umbilical vein endothelial cells (HUVECs) and human bone marrow stromal cells (HBMSCs) and on stimulation of vascularization and osteogenesis *in vivo* through combining co-cultures with CS containing scaffolds. Specifically, the effects of CS on the angiogenic growth factor VEGF, osteogenic growth factor BMP-2 and the cross-talks between VEGF and BMP-2 in the co-culture system were elucidated. Results showed that CS stimulated co-cultured HBMSCs (co-HBMSCs) to express VEGF and the VEGF activated its receptor KDR on co-cultured HUVECs (co-HUVECs), which was also up-regulated by CS. Then, BMP-2 and nitric oxide expression from the co-HUVECs were stimulated by CS and the former stimulated osteogenic differentiation of co-HBMSCs while the latter stimulated vascularization of co-HVUECs. Finally, the poly(lactic-co-glycolic acid)/CS composite scaffolds with the co-cultured HBMSCs and HUVECs significantly enhanced vascularization and osteogenic differentiation *in vitro* and *in vivo*, which indicates that it is a promising way to enhance bone regeneration by combining scaffolds containing silicate bioceramics and co-cultures of ECs and OCs.

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1. Introduction

In tissue engineering studies, it has been widely reported that biomaterials can affect behavior of single type of cells. For example, in bone tissue engineering research, many studies have shown that silicate bioceramics or bioglass could stimulate osteoblastic cells (OCs) differentiation [1–7]. Recently, it has been reported that silicate bioceramics or bioglass not only can stimulate osteogenic differentiation of OCs, but also be able to enhance proangiogenesis of endothelial cells (ECs) [4,8–12].

Interestingly, our recent studies showed that silicate biomaterials not only affected mono-type of cells but also affected interactions between different types of cells [13–15]. Calcium silicate (CS), which is a silicate bioceramic with simple composition of Si and Ca, has been found not only to stimulate proangiogenesis of mono-cultured human umbilical vein endothelial cells (HUVECs) but also to enhance proangiogenesis of HUVECs through affecting the interactions between HUVECs and human dermal fibroblasts (HDFs) in a direct contact co-culture system [14,15]. We further found that, when HUVECs alone were treated by CS extracts, the expression of vascular endothelial growth factor (VEGF) was stimulated, which subsequently activated

* Corresponding author. Shanghai Institute of Ceramics, Chinese Academy of Sciences, 1295 Dingxi Road, Shanghai 200050, China. Tel.: +86 21 52412804; fax: +86 21 52413903.
** Corresponding author. Shanghai 9th People's Hospital, Shanghai Jiao Tong University School of Medicine, 639 Zhizaoju Road, Shanghai 200011, China. Tel.: +86 13501909852; fax: +86 21 64397277.
E-mail addresses: drkailiu@126.com (K. Liu), Jchang@mail.sic.ac.cn (J. Chang).
[1] The two authors contributed to the work equally.

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VEGF's receptor KDR in HUVECs and initiated the angiogenesis pathway, in which autocrine effects played a critical role [14]. However, in the co-culture system of HUVECs and HDFs, CS extracts firstly stimulated abundant VEGF from co-cultured HDFs (co-HDFs), which subsequently activated expression of its receptor KDR from co-cultured HUVECs (co-HUVECs) and initiated the vascularization, in which paracrine effects played a key role, and the stimulation of vascularization by CS in co-culture system was significantly higher than that in HUVECs mono-culture [15]. These results suggest that the effects of biomaterials on single type of cells are different from that on co-culture systems, which more closely represent the real situation of tissue regeneration in which different type of cells are involved.

Previous studies have revealed an intimate relationship between bone vascular endothelium and osteoblastic cells [16,17]. Many studies have demonstrated that interaction between ECs and OCs is the prerequisite for vascularization during both intramembranous and endochondral ossification, and complex reciprocal cell communications have been reported in several *in vitro* studies [18–24]. Since biomaterials are always involved as cell carrier scaffolds in bone tissue engineering, it is interesting to reveal the role of silicate biomaterials in the interactions between OCs and ECs during tissue regeneration process, and the elucidation of the possible mechanism through which the silicates stimulate vascularization and osteogenesis *in vivo* where interactions between OCs and ECs naturally happen may provide information for designing optimal biomaterials for tissue engineering.

VEGF has been widely accepted as an important angiogenic growth factor and silicate biomaterials have been reported to enhance vascularization of ECs through stimulating the expression of VEGF from ECs or neighbor cells [8,14,15,18,23–25]. Furthermore, it has been widely accepted that BMP-2 is able to stimulate osteogenic differentiation and subsequently bone forming [26–29] and silicate biomaterials have also been reported to strongly affect the osteogenic differentiation of OCs through enhancing the BMP-2 expression [5,7]. However, so far, the behavior and role of BMP-2 in the interactions between OCs and ECs have rarely been studied. Besides, BMP-2 is also known to stimulate neovascularization in bone [30]. According to these reports, BMP-2 is not only an important growth factor for osteoblastic differentiation but may also be a critical growth factor for angiogenesis. Furthermore, some literature indicated that VEGF and BMP-2 might communicate with each other and affect osteogenesis and angiogenesis [31,32]. Therefore, our hypothesis is that BMP-2 may cross-talk with VEGF in the silicates mediated interactions between OCs and ECs during angiogenesis/vascularization and osteogenesis processes.

Therefore, in this study, we aimed to investigate effects of CS on the communication between HUVECs and HBMSCs, which finally affect the vascularization and osteogenesis in co-cultures. Specifically, we focused on investigating the roles of VEGF and BMP-2 in the interactions between HUVECs and HBMSCs and to elucidate the relationship between BMP-2 and VEGF, which are very critical for studying the mechanism through which the co-culture system stimulates vascularization and osteogenesis.

2. Materials and methods

2.1. Calcium silicate synthesis

CS powders were prepared by a chemical co-precipitation method [33]. The extracts of CS bioceramics were prepared according to the procedures reported in literature [34]. After extraction, CS extracts were diluted either with DMEM (GIBCO) + 10% FBS (Fetal Bovine Serum) (HyClone)+ 1% P/S (penicillin/streptomycin) (abbreviation in DMEM) or with Endothelial Cell Medium+5% FBS (Fetal Bovine Serum) + 1% ECGS (endothelial cell growth supplement) + 1% P/S (penicillin/streptomycin) (all from Sciencell, USA) (abbreviation in ECM) at the ratios of 1/64, 1/128 and 1/256. To determine the Ca, Si and P ion concentrations in bioceramic extracts, 5 ml diluted samples taken from the media for cell culture were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES; Optima 3000DV, Perkin Elmer, USA). DMEM and ECM were also analyzed by ICP-AES as control medium.

2.2. Cell isolation and culture

HUVEC and HBMSC were isolated according to previously described methods [22,23]. The obtained HUVECs and HBMSCs were cultured in ECM and DMEM, respectively. HUVECs at passages from 3 to 5 and HBMSCs at passages of 2 were used in this study. Cells were either mono-seeded or co-seeded at the densities of 20,000 HBMSCs/cm^2 and 40,000 HUVECs/cm^2. After cells were seeded for 12 h, cell culture media was changed. Mono-cultured HUVEC was cultured with ECM with different CS extracts and HBMSC was cultured with DMEM with different CS extracts. The media for co-culture is 1:1 ECM/DMEM with different CS extracts.

2.3. Vascularization and osteogenic differentiation of mono-cultured or co-cultured cells

To observe the vascularization of cells, the cultures were monitored under an inverted fluorescence microscope (Leica DMI 3000B, Germany) equipped with an excitation filter (490 nm), a dichroic mirror (505 nm) and a long-pass emission filter (530 nm). After being cultured for 1 day, a Live-Dead viability cytotoxicity kit (Invitrogen) was applied to stain cells and images were taken using a CCD camera (Leica DFC 420C) equipped with a fluorescence microscope. After being cultured for 3 days, the cells were immunofluorescence stained for von Willebrand Factor (vWF) using a rabbit anti-vWF primary antibody and an Alexa 488 goat anti-rabbit IgG secondary antibody (Invitrogen) to observe the tube formation of co-HUVECs. Briefly, after being cultured for determined time period, the cell layers were washed with Hank's buffered salt solution (HBSS, GIBCO) for at least three times and fixed with 4% (wt./vol.) paraformaldehyde (PFA) at 4 °C for 20 min. Cells were then permeabilized with methanol and blocked with HBSS containing 1% (wt./vol.) bovine serum albumin (BSA) for 1 h at 37 °C before incubation at 37 °C for 2 h in a primary antibody solution containing rabbit anti-vWF (diluted in HBSS-0.5% BSA at 1/100). For revealing the vWF, cells were washed with HBSS followed by incubation with Alexa 488 goat anti-rabbit IgG secondary antibody (Invitrogen). Finally, nuclei were stained by incubating the cells with 1 μg/ml 4-6-diamidino-2-phenylindole (DAPI) (FluoProbes) for 10 min at room temperature. The cells were then observed with an inverted fluorescence microscope and images were taken by a CCD camera. Five images were taken per well and tubules were manually counted and averaged over the five images.

To reveal the ALP expression in co-cultured cells, cells were stained after being cultured for 3 days according to the method described previously [22]. Briefly, cells were first washed with HBSS, fixed by 4% PFA for 15 min at room temperature, and then rinsed by deionized water. Thereafter, the cells were incubated in the Naphthol/Fastblue ready-to-use solution (Naphthol/Fastblue = 166 μl/4 ml, Sigma) in dark at 37 °C for 20 min according to the manufacturer's instruction. After incubation, cells were washed with HBSS before observation under an inverted microscope with bright field. Images were then taken by a CCD camera. ALP activity was detected by an alkaline phosphatase assay kit (abcam) according to the supplier's protocol. The kit uses *p*-nitrophenyl phosphate (*p*NPP) as a phosphatase substrate which turns yellow (max = 405 nm) when dephosphorylated by ALP. Finally, the ALP activity was expressed in nmol/ml/min.

2.4. Separation of HUVECs and HBMSCs after direct contact co-culture using magnetic beads

In order to measure the gene expression in co-HUVECs and co-HBMSCs, magnetic beads were applied to separate the co-cultured cells with magnetic beads combined with an antibody against CD31 (Invitrogen), a specific protein of endothelial cells, according to the method established by Guillotin et al. [35]. Briefly, after being cultured for 3 days, co-cultured cells were trypsinized and centrifuged, and the obtained cell pellet was resuspended in HBSS in a centrifuge tube. Then, magnetic beads were added into the solution according to the manufacture's instruction. After the solution being cultured for 30 min with agitation at 4 °C, the centrifuge tube was then placed on a stack with a magnet on one side for 5 min and the HUVECs with the beads were separated from the HBMSCs in the supernatant. The separated HUVECs and HBMSCs were named co-HUVEC and co-HBMSC, respectively.

2.5. Expression of VEGF, KDR, eNOS, NO, BMP-2 and ALP in mono-cultured or co-cultured cells

After the cells were cultured for 3 days, the amount of BMP-2 protein present in the culture media was also quantified by BMP-2 ELISA kits (Ray Biotec) according to the protocol provided by the supplier. Values for the amount of secreted BMP-2 are presented after subtraction of the amount of BMP-2 measured in media without cells.

To determine the NO synthesis in cells, diaminofluorescein-2 (DAF-2, Sekisui Medical) was chosen as a fluorescent indicator to perform the NO staining according

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