HUVECS-CONDITIONED MEDIUM HAS A BETTER POTENTIAL TO STIMULATE DIFFERENTIATION OF DENTAL PULP STROMAL CELLS TOWARD AN OSTEOLASTIC LINEAGE

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ABSTRACT

INTRODUCTION: One of the biggest challenges in tissue engineering technique is adequate vascularization to prevent tissue necrosis. This problem often arises in large bone defect reconstruction. During osteogenesis, vascularization involves cross-talk regulation between endothelial and osteoblast cells. It was the foundation of this study.

OBJECTIVES: To evaluate the effect of co-culture and conditioned medium of human umbilical vein endothelial cells (HUVECs) on the expression of collagen I, fibronectin proteins and alkaline phosphates (ALP) activity released by dental pulp cells (DPSCs) on 3D scaffold composed of hydroxyapatite/tricalcium phosphate/chitosan (HA/TCP/chitosan).

MATERIAL AND METHODS: The three main research group were (1) DPSCs co-cultured with various ratios of HUVECs, (2) DPSCs cultured with various concentrations of HUVECs-conditioned medium (CM), (3) DPSCs cultured with normal medium as the control. Culture media were collected at day 3, 5, 7 to examine the collagen type 1, fibronectin protein and ALP activity levels.

RESULTS: DPSCs-HUVECs indirect co-culture increased the protein expression of collagen I and fibronectin. However, the upregulation was higher when DPSCs were cultured with HUVECs CM. ALP was not changed in co-culture systems while it increased in the HUVECs CM group. The increase in three different markers tested in HUVECs CM compared to the co-culture system might be explained by the presence of signaling molecules in the HUVECs CM that stimulate DPSCs. Fibronectin expression was necessary for DPSCs' attachment to the 3D scaffold, while collagen I and ALP expressions might indicate the differentiation of DPSCs to osteoblasts.

CONCLUSIONS: HUVECs-conditioned medium has a better potential to be used to stimulate the differentiation of dental pulp cells towards an osteoblastic lineage, the condition that is necessary for the success of bone tissue engineering.

KEY WORDS: endothelial cells, dental pulp cells, scaffold, calcium phosphate, chitosan.

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INTRODUCTION

Bone is one of the highly vascularized tissues and the development of vascularization is critical for bone homeostasis and regeneration. Blood vessels mediate the transport of circulation cells, oxygen, nutrient and angiogenic molecules providing paracrine signals that promote the growth and differentiation of different tissues including bone [1]. Endothelial cells that reside in the blood vessel walls form new blood capillaries by the sprouting of existing vessels in the angiogenesis process and there is intimate communication between endothelial cells and bone cells to maintain the skeletal integrity [2, 3]. Cross-talk regulation from functional interaction between endothelial cells and osteoblasts during osteogenesis has been previously reported [2-6]. During intra-membranous bone regeneration, osteoblasts sense oxygen tension during the initial inflammatory phase of the healing process and respond to hypoxic conditions by activating the hypoxia-inducible factor α (HIF- α) pathway that promotes endothelial migration and proliferation [4]. Vascular endothelial growth factor (VEGF) is a key regulator for angiogenesis and it is critical for both bone development and regeneration. VEGF acts both on endothelial cells (ECs) to promote EC migration and proliferation and on osteoblast migration and proliferation through the regulation of osteogenic growth factors [5, 6].

In bone tissue engineering, one of the biggest challenges is to have adequate vascularization so as to prevent tissue necrosis. The maintenance of cell viability in the core of the cell-tissue construct depends on the invasion of existing blood vessels through angiogenesis [7]. Poor blood supply results in cell death due to lack of oxygen and nutrients and other soluble factors that are necessary for osteoblasts' proliferation and differentiation. It has been reported that oxygen diffusion to the surrounding tissue is limited to 150 μ m from the adjacent capillary vessels and the majority of cells located further away from the blood vessels will experience hypoxia [7]. The survival of cells and the scaffold construct therefore rely on the formation of an endogenous capillary network in response to angiogenic factors released by the hypoxic cells and the growth is approximately 100 nm per day [7]. To accelerate the vascularization of the cell-scaffold construct following *in vivo* implantation several approaches have been introduced such as incorporating endothelial cells to initiate the angiogenesis process [8-11]. Endothelial cells in a 3D scaffold could be used for large tissue reconstruction and transplanted *in vivo*. This would not only solve the problem of nutrient and oxygen diffusion to the inner part of the constructed bone tissue, but simultaneously it could stimulate osteogenesis through endothelial cells. As such, incorporating simultaneously multiple strategies of angiogenesis has been proposed to improve the success of bone tissue engineering [8-11].

Oral tissue origin mesenchymal stromal cells (MSCs) such as dental pulp stromal cells (DPSCs) have a great clinical potential and are relatively easy to harvest. One of the earliest reports of DPSCs demonstrated higher numbers of colony-forming cells and more bromodeoxyuridine positive cells than bone marrow derived MSC (BM-MSCs), indicating a larger clonogenic MSC population in DPSCs than in BM-MSCs [12]. Previous studies have reported the interaction of endothelial cells (ECs) with oral tissue origin mesenchymal stromal cells (MSCs) for odontogenesis and osteogenesis [13-16]. ECs' secretory product endothelin-1 (ET-1) influenced the differentiation of DPSCs whereby a higher level of odontoblastic differentiation-associated genes was observed [13]. The interaction between these two cells occurs via direct cell-cell contact and paracrine signaling. Other studies have reported a three-dimensional (3D) co-culture model in hydrogel; more extracellular matrix was deposited, and higher levels of vascularization and mineralization *in vivo* were seen than in the DPSCs monocultures [14]. Although a number of studies have reported interaction of ECs and DPSCs in direct and indirect co-culture models, the effect of EC-conditioned medium on DPSCs proliferation and differentiation is still unclear. EC-conditioned medium is known to contain numerous EC secreted proteins that could promote DPSCs differentiation [17]. In the present study, we aimed to evaluate the effect of EC-conditioned medium on DPSCs differentiation and compared the results with the effect of direct and indirect EC-DPSCs co-culture.

MATERIAL AND METHODS

MATERIAL

DMEM, fetal bovine serum (FBS), penicillin, streptomycin, fungizone, collagenase I, PBS, M200 medium supplemented with LSGS kit, PBS, were purchased from Gibco, California, USA. CD71, CD90, CD45 antibodies, CD31, V-CAM were from BD Sciences. Dispase from Roche, Indianapolis, USA. Glucose (Sigma, St Louis, USA). Quantichrom™ Alkaline Phosphatase Assay Kit were from BioAssay Systems, USA. Neutravidin HRP were from ThermoFisher Scientific, USA. Bradford total protein assay (Bio-Rad Protein Assay Kit, Bio-Rad, USA). HA/TCP/chitosan scaffold were manufactured in National Nuclear Energy Agency of Indonesia, Jakarta, Indonesia. Well-plate, culture flasks were from NUNC, Denmark. Trans-well PET membrane, 8.0 μ m were from Corning USA.

DENTAL PULP STROMAL CELLS ISOLATION

The study obtained ethical approval from the Dental Research Ethics Committee Faculty of Dentistry Universitas Indonesia. Dental pulp tissues were harvested from

FIGURE 1. Dental pulp stromal cells (DPSCs) and human umbilical vein endothelial cells (HUVECs). **A)** DPSCs showed their ability to form colony forming units (> 50 cells). **B)** HUVECs characterized by their morphological structure

three impacted teeth as previously reported [18]. Briefly, the teeth were crushed with a mortar and pestle, removed from the root canal by an extirpation needle and finely chopped with a sterile scalpel. The cells were dissociated from dental pulp tissue by enzymatic degradation with 4 mg/ml collagenase I (Gibco, California, USA) and 3 mg/ml dispase (Roche, Indianapolis, USA) in a 15 ml tube (BD Falcon, New Jersey, USA) at 37°C under 5% CO₂ in an incubator for 1 h. The process was terminated by adding complete DMEM medium supplemented with 20% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1.25 µg/ml Fungizone (Gibco, California, USA). The dental pulp cells were then cultured in a 6-well plate (Nunc, Roskilde, Denmark) until they reached about 90% confluency for approximately 14 days. 10⁷ cells were prepared for cell sorting by fluorescence-activated cell sorting (FACS) in a FACS Aria (BD Biosciences, USA) by using CD71, CD90, and CD45 antibodies (BD Biosciences). CD71- and CD90-positive cells and CD45-negative cells indicated the presence of DPSCs.

HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS ISOLATION

Endothelial cells were isolated from umbilical cord (human umbilical vein endothelial cells – HUVECs). Umbilical cords were obtained during breech deliveries with cesarean section at Budi Kemuliaan Maternity Hospital, Jakarta, Indonesia. All patients have signed informed consent.

Cord buffer transport medium containing 2 g/ml glucose (Sigma, St. Louis, USA) in PBS with 1% penicillin-streptomycin was used to store umbilical cords of approximately 15 cm. HUVECs isolation was performed within 4 h of umbilical cord collection. Umbilical veins were washed with sterile PBS with 2 cannulae fixed in both cord extremities to remove the remaining blood clot.

HUVECs cells were separated from the blood vessel wall by injecting 0.2% collagenase I (Gibco, California, USA) into the umbilical vein and incubated briefly for 7 minutes at 37°C in an incubator to avoid any contamination of fibroblast or smooth muscle cells. The cords were massaged gently to facilitate cell detachment and were washed with M200 medium supplemented with an LSGS kit containing fetal bovine serum, 2% v/v; 1 mg/ml hydrocortisone; 10 ng/ml human epidermal growth factor; 3 ng/ml basic fibroblast growth factor; and 10 mg/ml heparin (Gibco, California, USA).

HUVECs were cultured in a 6-well plate (Nunc, Roskilde, Denmark) for approximately 6 days until reaching 80% confluency.

HUVECs was identified microscopically by their polygonal phenotype and by flow cytometry using CD34, VCAM-1 antibodies for positive markers and CD45 antibody for negative marker (BD Biosciences, USA) [19, 20].

CELL CULTURES

DPSC and HUVECs passages 2-4 were used for the experiments (Figure 1A-B). DPSCs were cultured in osteogenic medium consist of: DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1.25 µg/ml Fungizone, 100 µg ascorbic acid, and 10 mM β-glycerophosphate, 10 nM dexamethasone. In the treatment group, DPSCs media were supplemented with 25%, 50%, 75%, 100% HUVECs-conditioned medium (day 5); DPSCs were co-cultured HUVECs with 1 : 1; 1 : 2; 1 : 4 HUVECs : DPSCs ratio in direct co-culture as well as indirect co-culture using a transwell insert system. Culture medium was collected at day 3, 5, 7 and the total protein was measured using Bradford total protein assay (Bio-Rad protein assay kit, Bio-Rad, USA). ALP assay was carried out using 10 µg of protein added to diethanolamine buffer and P-nitrophenyl phosphate. The absorbance value was read at 405 nm. Collagen type I protein and fibronectin protein were analyzed by ELISA assay. Briefly, a 96-well plate was incubated with

FIGURE 2. The effect of human umbilical vein endothelial cells (HUVECs) conditioned medium (CM) on collagen I, fibronectin and alkaline phosphates (ALP) activity released by dental pulp cells (DPSCs) were shown in Figure **A, C, E**. While the effect of DPSCs-HUVECs indirect co-culture on collagen I, fibronectin, ALP activity were shown in Figure **B, D, F**, respectively

goat polyclonal to type 1 collagen and goat polyclonal fibronectin overnight at 4°C. The following day, the wells were washed with PBS 3 times and Neutravidin HRP 1 : 20 000 in 1% BSA was added to the wells. The absorbance value was read at 405 nm.

STATISTICAL ANALYSIS

GraphPad Prism 6 for Mac OS X was used to calculate data, which were presented as mean and standard deviation. Normality was tested using the Shapiro-Wilk nor-

malinity test. Data were analyzed using the Kruskal-Wallis test, and significance was accepted when $p < 0.05$.

RESULTS

HUVECs-conditioned medium (CM) increased the expression of collagen I protein. The increase was seen at all CM concentrations tested with the highest found at CM 25% on day 5. In contrast, collagen I decreased after DPSC-HUVECs co-culture for 3 and 5 days and started to increase on day 7. A similar observation was seen for the effect of CM on fibronectin proteins, where it increased the most at CM 25% on day 5. The increase of fibronectin in the co-culture group was found on day 5, earlier than the increase of collagen I found on day 7. The activity of ALP was measured and it showed the upregulation on days 5 and 7 after CM treatment while no upregulation of ALP was detected in the co-culture system at all time points tested (Figure 2).

DISCUSSION

Angiogenesis is an important process for the survival and differentiation of cells in bone tissue engineering [1]. A delay in angiogenesis may impair the survival of the cell-tissue construct in the defect area and could result in a less ideal clinical outcome. Endothelial cells play a role in the maintenance of vascular homeostasis as well as controlling the secretion of molecules that control cell proliferation [2, 3]. Maintenance of angiogenesis in tissue engineering relies on the ingrowth of blood vessels from existing host vessels. As inadequate angiogenesis could disrupt the desired tissue regeneration, a potential strategy to compensate the limitation is to incorporate a growth factor-like compound that has the capacity to induce angiogenesis. In fact, many recent studies have proposed the potential of incorporating endothelial cells in a co-culture system to improve vascularization of the implanted cell-scaffold construct. Communication between endothelial and bone cells is crucial for controlling vascular supply during bone growth, remodeling, and repair. This study examined the interaction between HUVECs and human DPSCs. The effect of HUVECs on DPSCs differentiation towards an osteoblastic phenotype was studied by culturing them in two different types of HUVECs-DPSCs co-cultures, indirect contact and HUVECs-conditioned medium. Osteogenic markers of collagen I and ALP as well as fibronectin protein expression were evaluated at different time points. Our studies revealed that: (i) DPSCs responded earlier to HUVECs-conditioned medium compared to co-culture systems in terms of the secretion of collagen I and fibronectin; (ii) HUVECs-conditioned medium also promoted the ALP activity but not in HUVECs-DPSCs co-culture up to 7 days time point. These data highlight the potential of HUVECs-conditioned

medium as a stimulus for osteogenic differentiation of DPSCs.

Previous studies reported the effect of direct and indirect HUVECs-DPSCs co-cultures, enhancing the odontogenic potential of DPSCs as well as vasculogenic capacity of HUVECs in cell-cell contact culture [13-15]. Higher alkaline phosphatase (ALP) activity level, mRNA expression of ALP, bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP) genes and alizarin red staining were found in the co-culture group compared to the DPSCs alone. The study suggested that the higher odontogenic potential of DPSCs and vasculogenic capacity of HUVECs in the HUVECs-DPSCs co-culture system was facilitated by direct cell-cell contact, autocrine and paracrine stimulation. Our study tested the hypothesis that EC-conditioned medium has the capacity to promote DPSCs osteogenic differentiation as observed in the HUVECs-DPSCs co-culture system. Our study presented evidence that HUVECs-conditioned medium has a better potential to induce osteogenic differentiation of DPSCs. The presence of signaling molecules in the HUVECs-conditioned medium stimulates the differentiation of DPSCs. Previous studies have demonstrated that human ECs produce soluble mediators that enhance human osteogenic behavior [6]. VEGF has a possible role as an indirect regulator of osteoblasts operating via endothelial cell-derived osteogenic factors that modify osteoblasts. VEGF promotes IGF-I and endothelin release from ECs to modify osteoblast differentiation [6].

The effect of endothelial cell-conditioned medium has been tested in bone marrow-derived mesenchymal stromal cells (BM-MSCs) [21]. In contrast to our finding, EC-conditioned medium inhibit BM-MSCs' differentiation into mature osteoblasts. BM-MSCs are the most utilized MSCs and their characteristics are well studied [22]. The characteristics of BM-MSCs and DPSCs share many similarities and differences. Growth curves and colony formation assay indicated a higher proliferation rate of DPSCs compared to BM-MSCs. BM-MSCs have more adipogenic potential and less osteogenic potential compared to DPSCs [23, 24]. The differences in their characteristics might explain the contradictory results of BM-MSCs and DPSCs toward their differentiation. DPSCs provide a promising alternative source of MSCs for bone tissue engineering [25-27]. The conclusion of this study is that HUVECs-conditioned medium has the potential to be used to stimulate the differentiation of dental pulp cells to become osteoblasts, which is necessary for bone tissue engineering.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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