The Significance of SDF-1α-CXCR4 Axis in \textit{in vivo} Angiogenic Ability of Human Periodontal Ligament Stem Cells

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Periodontal ligament stem cells (PDLSCs) are multipotent stem cells derived from periodontium and have mesenchymal stem cell (MSC)-like characteristics. Recently, the perivascular region was recognized as the developmental origin of MSCs, which suggests the \textit{in vivo} angiogenic potential of PDLSCs. In this study, we investigated whether PDLSCs could be a potential source of perivascular cells, which could contribute to \textit{in vivo} angiogenesis. PDLSCs exhibited typical MSC-like characteristics such as the expression pattern of surface markers (CD29, CD44, CD73, and CD105) and differentiation potentials (osteogenic and adipogenic differentiation). Moreover, PDLSCs expressed perivascular cell markers such as NG2, α-smooth muscle actin, platelet-derived growth factor receptor β, and CD146. We conducted an \textit{in vivo} Matrigel plug assay to confirm the \textit{in vivo} angiogenic potential of PDLSCs. We could not observe significant vessel-like structures with PDLSCs alone or human umbilical vein endothelial cells (HUVECs) alone at day 7 after injection. However, when PDLSCs and HUVECs were co-injected, there were vessel-like structures containing red blood cells in the lumen, which suggested that Anastomoses occurred between newly formed vessels and host circulatory system. To block the SDF-1α and CXCR4 axis between PDLSCs and HUVECs, AMD3100, a CXCR4 antagonist, was added into the Matrigel plug. After day 3 and day 7 after injection, there were no significant vessel-like structures. In conclusion, we demonstrated the perivascular characteristics of PDLSCs and their contribution to \textit{in vivo} angiogenesis, which might imply potential application of PDLSCs into the neovascularization of tissue engineering and vascular diseases.

Keywords: angiogenesis, mesenchymal stem cells, periodontal ligament stem cells, perivascular cells, SDF-1α-CXCR4 axis

INTRODUCTION

Human teeth contain various types of stem cells and periodontal ligament stem cell (PDLSC) is one of representative stem cells residing in the periodontium (Seo et al., 2004). Functionally, PDLSCs are thought to regenerate damaged periodontium because PDLSCs can make periodontal ligament-like...
and cementum-like structures in vivo (Seo et al., 2004). PDLSCs have the expression pattern of specific surface antigens and in vitro differentiation potentials, which are similar to typical mesenchymal stem cells (MSCs) (Ivanovski et al., 2006; Nagatomo et al., 2006). Recently, pericyte-like characteristics of PDLSCs have been reported (Iwasaki et al., 2013), which is in accordance with reports suggesting that the origin of MSCs is the perivascular region (Crisan et al., 2008; Corselli et al., 2010).

Tissue engineering requires rapid vascular networks with the recipient’s vessels, which provides nutrients and oxygen, as well as discard exhausted materials (Jain et al., 2005). To solve the problem of vascularization, several solutions including the delivery of angiogenic factors have been suggested. However, there are limitations involved in the vascularization of thick engineered tissues (Isner and Asahara, 1999; Isner et al., 1999). Recently, it was suggested that co-injection of endothelial (progenitor) cells and perivascular cells could result in the formation of microvessels in vivo, which might be useful for tissue engineering (Au et al., 2008; Meler-Martin et al., 2007).

The stromal cell-derived factor 1 (SDF-1) is one of CXC chemokines via binding to CXCR4 for signal transduction (Aiuti et al., 1997; Mohle et al., 1998). The SDF-1α and CXCR4 axis is an important signaling pathway in neovascularization, including embryonic vasculogenesis and cancer (Petit et al., 2007; Teicher and Fricker, 2010). Recently, the beneficial roles of SDF-1α were reported in the neovascularization in cardiac infarct (Huang et al., 2011; Wang et al., 2012) and regeneration in spinal cord injury, which showed that the upregulated level of SDF-1 at the injury site could recruit stem cells (Jaerve et al., 2012). Moreover, SDF-1 is involved in the migration, survival, proliferation, and differentiation of stem cells (Zemani et al., 2008).

Recently, PDLSCs are reported to have perivascular characteristics (Iwasaki et al., 2013). In this study, we investigated whether PDLSCs could be a potential source of perivascular cells, which contributed to in vivo angiogenesis. Primarily isolated PDLSCs from the third molar showed typical MSC-like characteristics including surface antigens and differentiation potentials. To confirm the perivascular characteristics of PDLSCs, the expression of perivascular cell markers was verified by quantitative PCR and FACS analysis. The in vivo angiogenesis of PDLSCs was confirmed using in vivo Matrigel plug assay, and vessel-like structures could be formed by coinjection of PDLSCs and HUVECs. Finally, the involvement of the SDF-1α-CXCR4 axis between PDLSCs and HUVECs in in vivo angiogenesis was determined by CXCR4 antagonist, AMD3100.

**MATERIALS AND METHODS**

**Primary isolation and culture**

The usage of human teeth was approved by the Institutional Review Board (S-D20070004). Primary isolation and culture of PDLSCs were conducted according to the previous reports (Lee et al., 2011; Seo et al., 2004). Briefly, periodontal ligament tissues were extracted from human third molars. They were minced and incubated in 1 mg/ml of Collagenase type I (Gibco, USA) and 2.4 mg/ml of Dispase (Gibco) at 37°C for 1 h. After the inactivation of the enzyme via α-MEM (HyClone, USA) and 10% FBS (HyClone), the cells were washed two times with α-MEM (HyClone). Single-cell suspensions were maintained in α-MEM supplemented with 10% FBS and 1% Antibiotic-Antimycotic (Gibco). The medium was replaced every 3 days. When cells became 70% confluency, we conducted subculturing with 0.05% Trypsin/EDTA (Gibco). To acquire growth curve, the population doubling length (PDL) was calculated at each passage. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and cultured in EGM-2 (Lonza) until the sixth passage. All experiments were conducted using HUVECs from the sixth passage.

**FACS analysis**

The expression of surface markers was verified using FACS analysis. Cells at third passage were detached and stained with fluorescent-conjugated antibodies, which are listed in Supplementary Table 1. Between 1.0 × 10⁶ and 5.0 × 10⁵ cells were treated with antibodies for 30 min on ice. After washing, FACS data were acquired using a FACS Calibur (Becton Dickinson, USA), which were analyzed using FLOWJO (Tree Star, Inc., USA) software.

**In vitro differentiation**

Cells were grown to be confluent and cultured in the osteogenic and adipogenic differentiation medium. Osteogenic differentiation medium contained α-MEM and 5% FBS supplemented with 50 μg/ml L-ascorbic acid phosphate (Sigma-Aldrich, USA), 10 mM β-glycerophosphate (Sigma-Aldrich), and 1 mM dexamethasone (Sigma-Aldrich). Adipogenic differentiation medium included α-MEM and 5% FBS supplemented with 10 μg/ml insulin (Sigma-Aldrich), 0.5 mM isobutylmethylxanthin (Sigma-Aldrich), 50 μM indomethacin (Sigma-Aldrich), and 1 μM dexamethasone. The medium was replaced every 3 days. After 21 days of differentiation, cells were fixed with 10% neutral-buffered formalin at 37°C for 10 min. After washing, cells were stained with Alizarin red and Oil red O solution (all from Sigma-Aldrich).

**Quantitative PCR (qPCR)**

The total RNA of PDLSCs and HUVECs was prepared using an RNeasy Mini Kit (Qiagen, USA). For the synthesis of CDNA, 2 μg of total RNA was reverse-transcribed using SuperScript III (Invitrogen TM, USA) according to the manufacturer’s instruction. qPCR was carried out using each primer and THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan) in a CFX Connect Real-Time PCR Detection System (Bio-rad, USA). The information of each primer and annealing temperature (Tm) was provided in Supplementary Table 2.

**In vivo Matrigel plug assay**

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) (SNU-1010046). PDLSCs and HUVECs were mixed to be a total of 2.0 × 10⁶ cells at ratios of 1:0, 1:1, and 0:1 in 200 μl of ice-cold Phenol Red-free Matrigel (BD Bioscience, USA). The mixture was injected into 8-week-old immunodeficient mice (NIH-bg-nu-xid, Harlan Sprague-Dawley, USA). A mouse was injected by
one Matrigel implant subcutaneously using a 25-gauge needle. To inhibit the SDF-1α and CXCR4 axis, 10 μM of AMD3100 (Sigma-Aldrich) was added into the Matrigel plug before injection.

Histology
Mice were euthanized at day 3 and day 7 after injection to remove Matrigel plugs. The Matrigel plugs were incubated in 10% neutral-buffered formalin overnight. Slides were prepared and sectioned at 5 μm-thickness. Slides were deparaffinized and rehydrated. Slides were stained with hematoxylin and eosin (all from Sigma-Aldrich), and observed under microscope.

Immunofluorescent staining
Endogenous peroxidase was blocked using 10% hydrogen peroxide (Sigma-Aldrich) for 15 min. After washing, antigen retrieval was performed with pepsin for 20 min at 37°C. After washing, slides were blocked with 10% normal goat serum (Jackson Immunoresearch Lab., USA) for 30 min. The primary antibodies including rabbit anti-CD31 (Santa Cruz Biotechnology, USA) and mouse anti-α-smooth muscle actin (Santa Cruz Biotechnology) were treated to the slides for overnight at 4°C. After washing, secondary antibodies including Alexa 488-conjugated goat-anti mouse IgG (Invitrogen) and Alexa 594-conjugated goat-anti rabbit IgG (Invitrogen) antibodies, were applied to the slides for 1 hour at room temperature. DAPI (Sigma-Aldrich) was used for counterstaining. We observed slides under a Fluoview FV 300 (Olympus, Japan).

RESULTS
Primary isolation and characterization of PDLSCs
Primary isolation of PDLSCs was conducted following a previous report (Lee et al., 2011; Seo et al., 2004). In vitro characterization of PDLSCs was conducted at the third passage. PDLSCs had mesenchymal stem cell (MSC)-like bi-polar morphology (Fig. 1A). We did not observe growth regression (Fig. 1B), but at late passages, PDLSCs exhibited a more elongated morphology (data not shown). The expression of surface markers was verified by FACS. PDLSCs expressed MSC markers (CD29, CD44, CD73, CD90, and CD105). However, PDLSCs were negative for hematopoietic cell markers (CD14, CD34, CD45, CD117, and HLA-DR) and the endothelial cell marker CD31 (Fig. 1C). To confirm the differentiation potentials, PDLSCs were cultured to be confluent, and the culture medium was changed to osteogenic or adipogenic medium for 21 days. The results of Alizarin red staining revealed calcium deposits, suggesting osteogenic differentiation of PDLSCs (Fig. 1D). Oil red O stained lipid vacuoles, suggesting adipogenic differentiation of PDLSCs (Fig. 1E).

The perivascular characteristics of PDLSCs
The perivascular region is known to be enriched by stem cells, and it is considered to be the origin of MSCs (Crisan et al., 2008; Corselli et al., 2010). We analyzed the expression of perivascular cells markers in PDLSCs. As shown Fig. 2A, PDLSCs expressed NG2, α-smooth muscle actin (α-SMA),
platelet-derived growth factor receptor β (PDGFRβ), and CD146. According the results of qPCR, the expression of α-SMA was the highest, but that of NG2 was the lowest. FACS data revealed that PDLSCs expressed NG2, PDGFRβ, and CD146, but the expression level of them was different (Fig. 2B). Despite of the lowest mRNA level of NG2, we could observe the expression of NG2, which showed positive and negative populations. However, the expression of PDGFRβ was homogenous and that of CD146 showed broad range. These data indicated that PDLSCs had perivascular cell-like characteristics, suggesting that the origin of PDLSCs might be the perivascular region. Next, we performed functional involvement of PDLSCs in in vivo angiogenesis.

**In vivo angiogenic potential of PDLSCs**

PDLSCs with or without HUVECs were injected subcutaneously into immunodeficient mice. At day 7 after injection, the Matrigel plug was removed and analyzed by hematoxylin and eosin (H&E) staining. As shown Fig. 3A, in the single injection group of PDLSCs or HUVECs, we could not observe functional vessel-like structures. However, in the co-injection group of PDLSCs and HUVECs, functional vessel-like structures were formed in the Matrigel plug. Moreover, we could observe erythrocytes and leukocytes in the lumen of newly formed vessels, suggestive of anastomosis with host circulatory system. Then, we analyzed the location of injected PDLSCs and HUVECs by staining with CD31 and α-SMA. The expression of α-SMA and CD31 was localized around vessel-like structures, which were formed by PDLSCs and HUVECs (Fig. 3B).

**The involvement of SDF-1α and CXCR4 axis in in vivo angiogenesis**

The SDF-1α-CXCR4 axis is known to regulate neovascularization in development and regeneration (Petit et al., 2007). qPCR data revealed that the expression of SDF-1α was found to be highly upregulated in PDLSCs compared to HUVECs (Fig. 4A). In contrast, the expression of CXCR4 is highly upregulated in HUVECs compared to PDLSCs (Fig. 4A). These contradictory expression patterns suggested reciprocal interactions between PDLSCs and HUVECs. To investigate the contribution of the SDF-1α-CXCR4 axis to in vivo angiogenesis, AMD3100, which is a known CXCR4 antagonist, was mixed with PDLSCs and HUVECs in a Matrigel plug.
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**Fig. 4. The involvement of the SDF-1α and CXCR4 axis in *in vivo* angiogenesis by PDLSCs and HUVECs.** The expression of SDF-1α and CXCR4 was determined by qPCR. (A) PDLSCs expressed SDF-1α, but CXCR4 expression was not detected. In contrast, HUVECs expressed CXCR4, but SDF-1α expression was not detected. To confirm the functional involvement of the SDF-1α and CXCR4 axis in *in vivo* angiogenesis, AMD3100, a CXCR4 antagonist, was mixed with the Matrigel plug. (B) At day 3 after injection, there were no obvious vessel-like structures in the AMD3100-treated group compared to the control group. (C) At day 7 after injection, there were no vessel-like structures in the AMD3100-treated group compared to the control group.

**DISCUSSION**

PDLSCs are multipotent stem cells derived from the periodontium that support teeth throughout life. However, despite the multipotency and beneficial effects of PDLSCs, such as immune modulatory capacity (Wada et al., 2009; Chen and Jin, 2010; Maeda et al., 2011; Zhu and Liang, 2015), the applications of PDLSCs have been limited to the regeneration of the periodontium and bone formation (Wada et al., 2009; Chen and Jin, 2010; Maeda et al., 2011; Zhu and Liang, 2015). In this study, we investigate the feasibility of PDLSCs as a perivascular cell source in tissue engineering and other vascular diseases.

Recently, it was reported that MSCs originate from the perivascular region (Crisan et al., 2008). A previous report suggests that perivascular regions are also the origin of dental pulp stem cells because of the perivascular localization and the expression of perivascular markers (Shi and Gronthos, 2003). Although PDLSCs are isolated from the periodontium, periodontium also contains many vessels, which suggests that PDLSCs may originate from the perivascular region and have pericyte-like characteristics. Although pericyte-specific markers have not been identified, the combined expression of various pericyte markers in PDLSCs suggested that they might have pericyte characteristics and that they might originate from the perivascular region. Recently, the expression of pericycle markers in PDLSCs was reported, and, in this study, we showed that PDLSCs were positive for perivascular cell markers including α-SMA, NG2, PDGFRβ, and CD146. Interestingly, primarily isolated PDLSCs expressed different levels of pericycle markers. These data suggested that PDLSCs are comprised of a heterogeneous population.

Perivascular cells, also called as pericytes, are located near endothelial cells and interact reciprocally to regulate development, stabilization, maturation, and remodeling of blood vessels (Armulik et al., 2005; Gaengel et al., 2009). We and
others have shown the expression of pericyte markers in PDLSCs, but their functional roles in vivo have not yet been identified. The in vivo Matrigel plus assay is useful to confirm the in vivo angiogenic potential of PDLSCs as a perivascular cell source. In our study, the injection of PDLSCs alone or HUVECs alone did not result in the formation of vessel-like structures in vivo. This result is in accordance with previous reports that suggested the necessity of co-injection with endothelial cell sources (Kim et al., 2016; Melero-Martin et al., 2008). However, when PDLSCs and HUVECs were co-injected into immunodeficient mice, vessel-like structures were readily formed and host blood cells were observed in the lumen. Moreover, immunofluorescent data revealed that PDLSCs co-localized within the perivascular region near HUVECs. These data suggested that PDLSCs could have a functional role as perivascular cells for in vivo angiogenesis and reciprocal interactions between PDLSCs and HUVECs need to be identified.

The effects of SDF-1α-CXCR4 axis have been reported in dental pulp stem cells, which are involved in the proliferation, differentiation, and recruitment (Gong et al., 2010; Jiang et al., 2008; 2012). SDF-1α increased the proliferation of and stimulated the migration of PDLSCs (Du et al., 2012). In our results, PDLSCs showed contradictory expression pattern of SDF-1α and CXCR4 which is in accordance with a previous report (Trubiani et al., 2008). These reports suggest important roles for SDF-1α in PDLSCs. In contrast, HUVECs showed high expression of CXCR4 but low expression of SDF-1α. This suggested functional roles of the SDF-1α and CXCR4 axis between PDLSCs and HUVECs. Actually, treatment showed high expression of CXCR4 but low expression of SDF-1α-CXCR4 axis between PDLSCs and HUVECs. These reports suggest the necessity of co-injection with endothelial cell sources (Kim et al., 2016; Melero-Martin et al., 2008). However, when PDLSCs and HUVECs were co-injected into immunodeficient mice, vessel-like structures were readily formed and host blood cells were observed in the lumen. Moreover, immunofluorescent data revealed that PDLSCs co-localized within the perivascular region near HUVECs. These data suggested that PDLSCs could have a functional role as perivascular cells for in vivo angiogenesis and reciprocal interactions between PDLSCs and HUVECs need to be identified.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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