Int J Clin Exp Pathol 2016;9(11):11262-11268
www.ijcep.com /ISSN:1936-2625/IJCEP0038502

Original Article

Effect of hypoxia on pluripotent differentiation of periodontal ligament stem cells and related mechanisms

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Received August 22, 2016; Accepted September 29, 2016; Epub November 1, 2016; Published November 15, 2016

Abstract: High incidence of periodontal disease occur in plateau regions, as hypoxia may affect biological feature of stem cells including periodontal ligament stem cell (PDLSCs) differentiation. This study thus tested activity of alkaline lipid phosphatase (ALP) and expression level of lipogenesis related gene, in an attempt to illustrate the effect of hypoxia on pluripotent differentiation on PDLSCs, plus an initial investigation of related mechanisms. Isolated PDLSCs were used to study the effect of hypoxia environment on pluripotent differentiation. ELISA was used to test levels of ALP in culture supernatants under different oxygen concentration. Real-time fluorescent quantitative PCR was used to measure mRNA level of PPARγ and LPL. Western blot was employed to quantify expression level of p38/MAPK and MAPK/ERK signal molecules. MPAK inhibitor was further employed to test the role of p38 or ERK in hypoxia-induced pluripotent differentiation potency of PDLSCs. Hypoxia group had significantly higher ALP activity, and lower expression level of PPARγ and LPL ($P<0.05$ compared to control group). No significant difference existed of total p38 or ERK1/2 of PDLSCs under different oxygen concentrations. Hypoxia group had remarkably elevated expression of phosphorylated p38 and ERK1/2 ($P<0.05$). p38 inhibitor significantly inhibited ALP activity, and mRNA expression level of PPARγ and LPL, while ERK inhibitor potentiated their expression level. Co-treatment of p38 and ERK inhibitor enhanced levels of these signal molecules ($P<0.05$). Hypoxia condition inhibits osteogenesis and lipogenesis differentiation potency of PDLSCs, possibly via p38/MAPK and ERK/MAPK signal pathways.

Keywords: Hypoxia, periodontal ligament stem cells, lipogenesis differentiation, osteogenesis differentiation

Introduction

Periodontal ligament is connective tissues with high fibrosis and vascularization, and is one important component of periodontal tissue to provide oxygen [1]. It has high potency of differentiation, and is the stress force center in orthodontic treatment due to special anatomical location [2]. Under stress stimuli or endogenous disease signal, specific cells in the microenvironment of periodontal ligament tissues can differentiate into osteocytes, adipocytes, fibroblasts or cementoblast. These functional cells play important roles in self-renewal and injury repair of periodontal tissues [3]. Therefore periodontal ligament is critical for tissue engineering reconstruction for periodontal disease.

Various evidences indicated stem cells inside periodontal ligament as the reason for tissue regeneration. In 2004, Seo et al for the first time identified and defined periodontal ligament stem cells (PDLSCs) from periodontal ligament tissues [4]. As one adult stem cell, PDLSCs has potencies of highly proliferation, self-renewal and pluripotent differentiation. Under *in vitro* induction culture, they can differentiate into osteoblast, lipoblast, chondroblast and neuron-like cells.

Seo et al also found that the function of PDLSCs was under the governing of multiple factors, including external stimuli and body immune status [4]. Various epidemiology surveys found significantly higher rate of periodontitis in people lived on plateau regions with >4000 m altitude

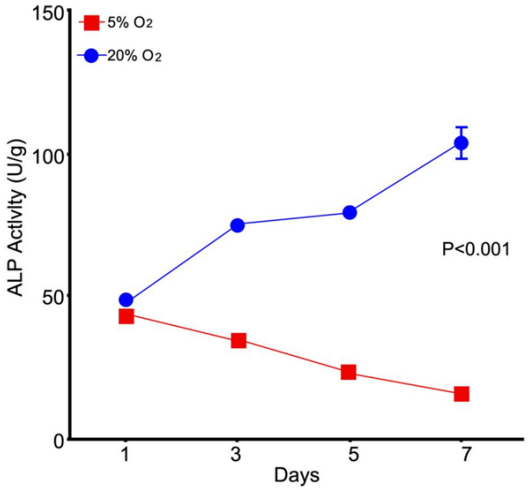

Figure 1. Hypoxia and ALP activity in PDLSCs.

compared to residents of plain regions [5]. Animal study also found hypoxia could induce severe periodontitis [6, 7]. Various studies showed that hypoxia affected biological property of stem cells, for example, hypoxia facilitated the differentiation of lipoblast toward chondrocytes [8, 9]. Other study however believed that hypoxia could inhibit lipoblast or osteoblast differentiation [10]. Moreover, hypoxia has effects on the differentiation of both neural stem cells [11] and human mesenchymal stem cells [12]. We thus speculated that hypoxia might affect differentiation of PDLSCs, thus causing elevation of periodontitis incidence in plateau residents. This study measured ALP activity and mRNA expression levels of lipoblast differentiation related genes PPARγ and LPL, in order to illustrate the influence of hypoxia on pluripotent differentiation of PDLSCs.

Mitogen activating protein kinase (MAPK) signal pathway is one of the most important signal pathways inside body, as its activation can regulate a series of cellular activities such as proliferation, differentiation and apoptosis. Previous studies showed the involvement of activation of p38/MAPK and MAPK/ERK signal pathways and osteoblast differentiation of stem cells [13, 14]. Therefore this study also measured the expression level of p38/MAPK and MAPK/ERK signal pathway related proteins in PDLSCs under hypoxia condition, in order to investigate the effect of hypoxia on PDLSCs differentiation.

Materials and methods

Reagents and equipment

Human PDLSCs culture: PDLSCs were previously isolated in our research group. In brief, PBS (Zhongshan, China) containing penicillin-streptomycin was used to rinse tooth tissue repeated, followed by the separation of periodontal tissues from middle 1/3 region. Tissues were cut into small pieces (1 mm^3) using sterile scissors and were transferred to centrifuge tube. After 250 g centrifugation for 5 min, supernatant was saved with the addition of type II collagenase (Sigma, US) for 1.5 h digestion under 37°C. By filtration in 70 μm filter, supernatant after 1000 rpm for 5 min was discarded for preparing single cell suspension. Cells were repeatedly rinsed and centrifuged by DMEM medium (Gibco, US) containing 10% FBS and penicillin-streptomycin, and were cultured at 1×10^4 per ml density in DMEM medium containing 10% FBS and penicillin-streptomycin in 37°C incubator (Thermo, US) with 5% CO_2. Cells were observed under a microscope. PDLSCs were then isolated and cultured by limited dilution method in 7°C incubator with 5% CO_2. Oxygen levels of control group and hypoxia group were set as 20% and 5%, respectively.

ELISA for measuring alkaline phosphatase activity

PDLSCs were cultured until reaching 80% confluence, and were digested in 0.25% trypsin. After rinsing in PBS for three times, PDLSCs were counted and collected. Cells were adjusted into 1×10^4 per ml by fresh medium and were seeded into 96 well plates. With cultured under normal (20% oxygen) and hypoxia (5% oxygen) conditions for 5 days, cells were lysed by TritonX-100 (Fanke, China). ELISA (Kaibo, China) was used to measure ALP activity following manual instruction. Optical density (OD) values at 450 nm were measured.

Real-time fluorescent quantitative PCR measuring PPARγ and LPL

PDLSCs were cultured under hypoxia or normal conditions. Cells were cultured, rinsed in PBS for three times and adjusted to 1×10^7 per ml. Equal volume of Trizol (Invitrogen, US) was used to extract total RNA from cells. The mixture was incubated on ice for 10 min, with the addition of

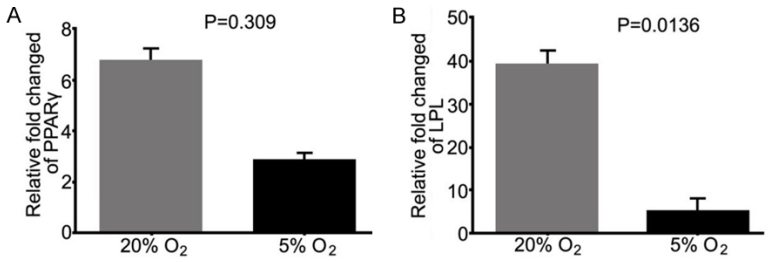

Figure 2. Hypoxia and mRNA level of PPARγ and LPL.

200 μl CCl_3, followed by 5 min mixture and cold centrifugation (12000 rpm, 15 min). The supernatant was saved and mixed with equal volume of isopropanol for 10 min incubation. With centrifugation (12000 rpm, 8 min), the supernatant was discarded. RNA precipitations were washed in pre-cold 70% ethanol, and were air dried. Protein-nucleic acid analyzer was used to test RNA purity and concentration, accompanied with 1% agarose gel electrophoresis (Sigma, US) for RNA integrity. 1 μg RNA was used for synthesis of cDNA using reverse transcription kit (Takara). Real-time fluorescent quantitative PCR system (Takara, Japan) was prepared including 5 μl 2XSYBR Green Mix, 0.5 μl cDNA, 0.5 μl primer (10 μM), and 4 μl ddH2O. The reaction conditions were: 95°C pre-denature for 10 min, followed by 40 cycles each containing 95°C denature for 15 s and 60°C annealing plus elongation for 60 s. PCR (ABI, US) was performed on a fluorescent quantitative cycler in triplicates for each sample and using GAPHD as the internal reference gene. Primer sequences were: GAPDH-forward, 5'-CGGGA AGCTT GTCAT CAATG G-3', GAPDH-reverse, 5'-GGCAG TGATG GCATG GACTG-3'; PPARγ-forward, 5'-CAGTT ACGTG GAAAA CAGAG TG-3', PPARγ-reverse, 5'-CGAAG ACATC ATCAT CTTAC AATCG A-3'; LPL-forward, 5'-ACATG CCTTT GCATA GCATG-3', LPL-reverse, 5'-TGAGA ACCCC GGAAG AATGA C-3. Semi-quantitative analysis of mRNA expression level was performed by $2^{-\Delta\Delta Ct}$ method.

Western blot for protein expression level

PDLSCs were cultured under hypoxia or normal conditions. Cells were collected and mixed with pre-cold RIPA lysis buffer (Beyotime, China) for iced incubation for 30 min. With centrifugation (12000 rpm, 25 min), supernatant was collected to obtain total protein solution. Protein concentration was measured by BCA kit (Shenneng, China) following manual instruction. 20 μg total proteins were diluted in 125 μl distilled water, and were mixed with 125 μl loading buffer Laemmli (containing 54 mg/ml dithiothreitol). Proteins were denatured at 100°C for 10 min. SDS-PAGE was prepared to separate loaded protein samples under electrophoresis (180 V, vertical field, 1 h). The gel was then transferred to the membrane (Millpore, US) (100 mA, 80 min). The membrane was blocked in 5% defatted powder, and was incubated in primary antibody for p38, p-p38, ERK1/2, p-ERK1/2 or β-actin for 4°C overnight incubation. The membrane was washed in PBST for five times (10 min each), followed by 1 h incubation in HRP (Kangcheng, Chinia)-labelled secondary in 37°C chamber. After five times of TBST rinsing (10 min each), excess fluids were removed from the membrane, which was incubated with chromogenic substrate at room temperature for 2~3 min. Development was performed in a dark room for exposing the film (Fuji, Japan).

Statistical method

SPSS19.0 was used for statistical analysis. Those data fitted normal distribution were presented as mean ± standard deviation. Student t-test compared means between two groups. A statistical significance was defined when $P<0.05$.

Results

Effects of hypoxia on osteoblast potency of PDLSCs

As one secretory enzyme of osteoblast, the expressional activity of ALP is one significant feature of osteoblast differentiation. This study thus investigated ALP expression level in PDLSCs to illustrate the influence of ALP on their osteoblast differentiation potency. ELISA method was used to test ALP activity (**Figure 1**). Via monitoring ALP release level at various time points, significant difference occurred between normal and hypoxia conditions since day 3. With elongated incubation time, such difference became more significant. In hypoxia group, ALP activity was further decreased ($P<0.001$ compared to control group).

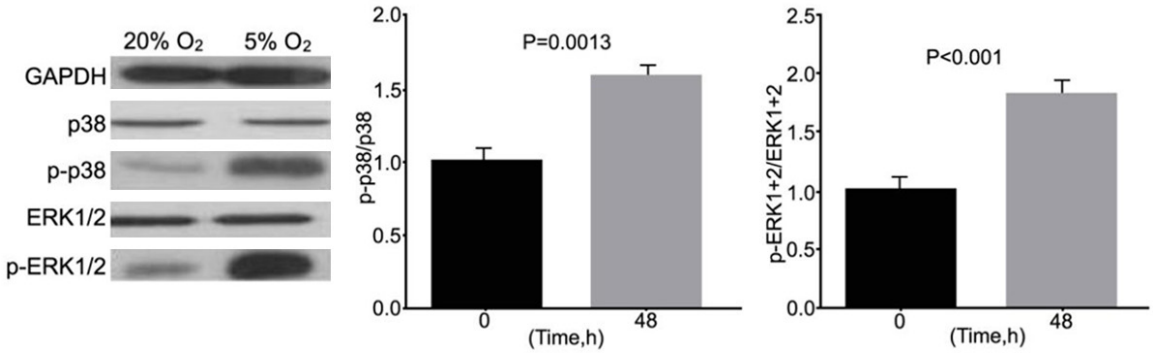

Figure 3. Hypoxia-cultured PDLSCs activated p38/MAPK and MAPK/ERK signal pathways.

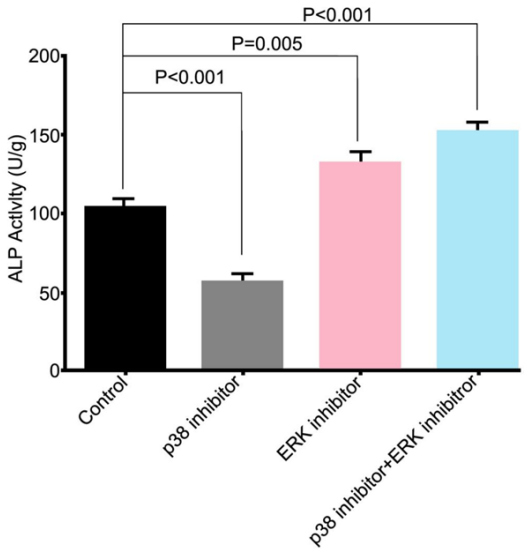

Figure 4. Effect of ERK inhibitor or p38 inhibitor on ALP expression.

Influences of hypoxia on lipoblast differentiation potency of PDLSCs

To illustrate the effect of hypoxia on lipoblast differentiation potency of PDLSCs, real-time fluorescent quantitative PCR was employed to test mRNA levels of lipoblast differentiation related genes PPARγ and LPL. As shown in **Figure 2**, at day 21 of cell culture, PPARγ and LPL mRNA levels in hypoxia group was significantly lower (P<0.05 compared to control group).

Hypoxia activated p38/MAPK and MAPK/ERK signal pathways

It has been reported that pluripotency of PDLSCs was correlated with p38/MAPK and MAPK/ERK signal pathways. We thus measured expression levels of related protein by Western blotting after cultured under different oxygen concentrations for 48 h. As shown in **Figure 3**, no significant difference existed of total p38 or ERK1/2 levels between PDLSCs cultured under different oxygen concentrations. Phosphorylated p38 and ERK1/2 levels, however, were significantly elevated in hypoxia group (P<0.05 compared to control group).

Inhibition of osteoblast differentiation ability of PDLSCs under hypoxia was correlated with p38/MAPK and MAPK/ERK signal pathways

To understand whether the activation of MAPK pathway under hypoxia condition could affect osteoblast differentiation ability of PDLSCs, we studied the effect of hypoxia culture on osteoblast differentiation potency via pre-treatment of PDLSCs by p38 inhibitor or ERK inhibitor. As shown in **Figure 4**, p38 inhibitor significantly inhibited the expression of ALP, which is one osteoblast marker for PDLSCs (P<0.001). In contrast, ERK inhibitor significantly elevated ALP expression (P=0.005). The co-treatment of p38 inhibitor and ERK inhibitor, however, remarkably elevated ALP expression (P<0.001).

Inhibition of lipoblast differentiation potency of PDLSCs by hypoxia was correlated with p38/MAPK and MAPK/ERK pathways

To study the effect of MAPK pathway activation of PDLSCs under hypoxia condition on their lipoblast differentiation potency, we further studied the effect of hypoxia condition on lipoblast differentiation ability of PDLSCs after pre-treatment cells using p38 inhibitor or ERK inhibitor. As shown in **Figure 5**, p38 inhibitor significantly suppressed the expression of lipo-

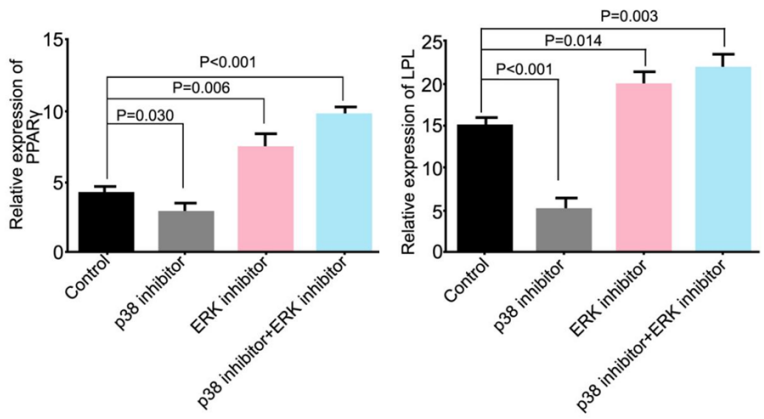

Figure 5. Effects of ERK inhibitor or p38 inhibitor on lipoblast gene PPARγ and LPL expression.

blast genes PPARγ and LPL ($P<0.05$ compared to control group). In contrast, ERK inhibitor remarkably potentiated PPARγ and LPL expression ($P<0.05$). Those cells treated by both p38 and ERK inhibitor had significantly elevated expression of PPARγ and LPL ($P<0.05$).

Discussion

Elevated expression of ALP can be observed in osteoclast and periodontal bone cells. As one important bio-mineralization marker protein, and index for initial phase of osteoblast differentiation, ALP is one of early features of osteoblast differentiation, and is related with early phase of osteoblast differentiation [15]. Therefore ALP is widely used to evaluate mineralization and osteoblast differentiation of different tissues/cells. This study thus measured ALP expressional activity of PDLSCs, to illustrate the effect of hypoxia on PDLSCs osteoblast differentiation. Results of this study showed significantly depressed ALP activity in hypoxia group, with more significant difference after elongated hypoxia treatment, indicating that hypoxia could inhibit osteoblast differentiation of PDLSCs. Wu et al and Yu et al also found the inhibition of osteoblast differentiation of PDLSCs by hypoxia condition [1, 16, 17], as consistent with our results.

Moreover, this study detected mRNA level of lipoblast related genes PPARγ and LPL [18], in order to identify the influence of hypoxia environment on lipoblast differentiation of PDLSCs Results showed that remarkably decreased mRNA levels of PPARγ and LPL in hypoxia group, suggesting that hypoxia also inhibited lipoblast differentiation ability of PDLSCs. Although hypoxia was known to inhibit pluripotency of PDLSCs, currently no report has mentioned the involvement of hypoxia in lipoblast differentiation by PDLSCs.

Previous studies found important roles of MAPK signal pathway family members, including p38 and ERK, in the differentiation of bone marrow mesenchymal stem cells into osteoblast and related cell division and proliferation [19-21]. Hypoxia environment can activate p38/MAPK and MAPK/ERK signal pathways [22]. This study thus also tested the expression level of p38/MAPK or MAPK/ERK signal pathway related proteins. Our results showed significantly elevated phosphorylated p38 and ERK1/2 expressions of PDLSCs under hypoxia condition, indicating the activation of p38/MAPK and MAPK/ERK pathway. The pre-treatment using p38 or ERK inhibitor on PDLSCs showed that p38 inhibitor could inhibit ALP activity, and expression level of PPARγ and LPL mRNA, whilst ERK inhibitor significantly enhanced ALP activity and mRNA expression level of PPARγ and LPL. The co-treatment using both p38 and ERK inhibitor showed remarkably elevated PPARγ and LPL mRNA levels, plus higher ALP activity. These results collectively suggested that hypoxia could inhibit osteoblast and lipoblast differentiation potency of PDLSCs via regulating balance between p38/MAPK and MAPK/ERK signal pathways. Wu et al also found the effect of hypoxia on osteoblast differentiation of PDLSCs via regulating p38/MAPK and MAPK/ERK signal pathways [1], as consistent with our results.

In summary, our data showed inhibition of pluripotent differentiation abilities of PDLSCs under hypoxia environment. Such effect on pluripotency was under the regulation on p38/MAPK and MPAK/ERK signal pathway balance. Such inhibited pluripotency of PDLSCs under hypoxia environment might be one reason for high incidence of plateau population. However, due to the complicated mechanism of inhibition on PDLSCs pluripotency, further studies are required.

Conclusion

Hypoxia environment could inhibit osteoblast and lipoblast differentiation potencies of PDLSCs. The balance between p38/MAPK and ERK/MAPK pathways might participate in the regulation of hypoxia on PDLSCs differentiation.

Acknowledgements

Research supported by the Key Projects in the National Science & Technology Pillar Program during the Twelfth Five-year Plan Period (NO. CWS11J320).

Disclosure of conflict of interest

None.

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