

Research Article

Open Access

Qing Wang, Gang Ding*, Xin Xu*

Periodontal ligament stem cells regulate apoptosis of neutrophils

DOI 10.1515/med-2017-0004

received October 18, 2016; accepted January 15, 2017

Abstract: Periodontal ligament stem cells (PDLSCs) are promising cell resource for the cell-based therapy for periodontitis and regeneration of bio-root. In this study, we investigated the effect of PDLSCs on neutrophil, a critical constituent of innate immunity, and the underlying mechanisms. The effect of PDLSCs on the proliferation and apoptosis of resting neutrophils and IL-8 activated neutrophils was tested under cell-cell contact culture and Transwell culture, with or without anti-IL-6 neutralizing antibody. We found that PDLSCs could promote the proliferation and reduce the apoptosis of neutrophils whether under cell-cell contact or Transwell culture. Anti-IL-6 antibody reduced PDLSCs-mediated inhibition of neutrophil apoptosis. IL-6 at the concentration of 10ng/ml and 20ng/ml could inhibit neutrophil apoptosis statistically. Collectively, PDLSCs could reduce the apoptosis of neutrophils via IL-6.

Keywords: Periodontal ligament stem cells; Neutrophil; Interleukin-6; Apoptosis

1 Introduction

Mesenchymal stem cells (MSCs) possess the self-renewal and multi-potent differentiation capabilities, and are easily isolated and cultured *in vitro*. Under appropriate culture conditions, MSCs can be induced into osteoblast, adipocyte, and chondrocyte, thus making them favorable candidate for cell-based treatment [1]. Until now, MSCs can be isolated and characterized from a variety of human tissues, including, but not limited to bone marrow, adipose tissue, umbilical cord, periodontal ligament, etc. Originating from periodontal ligament tissues, periodontal ligament stem cells (PDLSCs) are regarded as excellent seed cells for the treatment of periodontitis and regeneration of bio-root [2-4]. It is unavoidable that there is close contact between the MSCs and polymorphonuclear neutrophils (PMN) when MSCs are used for *in vivo* transplantation [5]. However, the effect of PDLSCs on PMN remains unknown. In this study, we investigated the effect of PDLSCs on PMN and underlying mechanisms.

2 Materials and methods

2.1 Cell culture

All protocols for the handling of human tissue were approved by the Research Ethics Committee of Shandong University, Jinan, China.

PDLSCs: Normal human impacted third molars were collected from 18-26-year-old patients at the Department of Oral and Maxillofacial Surgery, Shandong University School of Stomatology after the patients gave informed consent. The periodontal ligament from the middle third of the extracted molar root was separated gently from the surface of the root, and PDLSCs were isolated and cultured as described previously [2, 6]. PDLSCs were incubated in 25 cm² culture flasks (Costar, Cambridge, MA, USA) containing alpha-modification of Eagle's medium (GIBCO; Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (GIBCO), 100 µmol/l ascor-

*Corresponding author: **Gang Ding**, Department of Stomatology, Yidu Central Hospital, Weifang Medical University, Linglongshan South Road No.4138, Qingzhou262500, Shandong Province, People's Republic of China. E-mail: dentistdg@sina.com

Xin Xu, Department of Implantology, Shandong Provincial Key Laboratory of Oral Biomedicine, Shandong University School of Stomatology, Wenhua West Road No.44-1, Jinan250012, Shandong Province, People's Republic of China. E-mail: xinxu@sdu.edu.cn

Qing Wang, Department of Implantology, Shandong Provincial Key Laboratory of Oral Biomedicine, Shandong University School of Stomatology, Jinan250012

Qing Wang, Department of Dentistry, Weifang People's Hospital, Weifang261000, Shandong Province, People's Republic of China

bic acid 2-phosphate (WAKO, Tokyo, Japan), 2 mmol/l glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). The cells used in this study were at the 3-4 passage stage and could differentiate to osteoblasts and adipocytes *in vitro* (data not shown).

PMN: Venous blood of 20ml from healthy volunteers was anti-coagulated by heparin (10U/ml) and diluted by Hank's balanced salt solution (Invitrogen) at the ratio of 1:1. Then the diluted blood was carefully layered onto 5ml Ficoll (1.077 g/ml; Dingguo, Beijing, China) for centrifugation at 1,000 rpm for 10 min. After plasma and the mononuclear cell layer were discarded, the erythrocytes were removed by 5% dextran followed by hypotonic lysis in bidistilled water for 10s as described previously [7]. PMN were then washed 3 times and resuspended in RPMI1640 (GIBCO) supplemented with 10% fetal bovine serum, 2mmol/l glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 20mol/l HEPES (Invitrogen). Neutrophils were about 96% pure on average as determined by morphologic analysis.

2.2 Examination of the proliferation of PMN

PDLSCs of 1.0×10^4 , 5.0×10^4 , or 2.5×10^5 were cultured in triplicate in 96-well U-bottomed plates at 37°C in a humidified atmosphere supplemented with 5% CO₂. Two hours later, the supernatant was removed and allogeneic PMN (5.0×10^4) were added. The cells were cultured for 24 hours in 0.2 ml RPMI-1640 at 37°C with 5% CO₂. Then the PMN were collected and Cell Counting Kit-8 kits (Dojindo Laboratories, Japan) were used to examine the proliferation of PMN. The absorbance at 450 nm was measured on a Microplate Spectrophotometer (Molecules Devices, Sunnyvale, CA, USA). In other assays, IL-8 (10ng/ml; Sigma-Aldrich, St. Louis, MI, USA) were added into the culture system as above mentioned at the same time PDLSCs and PMN were co-cultured. The cells were cultured in 0.2 ml RPMI-1640 at 37°C with 5% CO₂, and PMN proliferation was measured as described above 24 hours later.

2.3 Detection of the apoptosis of PMN

The co-culture of PDLSCs and PMN (at the ratio of 1:5, 1:1, and 5:1) was established for 24 hours as above mentioned. Then PMN were collected and the percentage of apoptotic PMN was evaluated using the Annexin V-Fluos staining kit (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's instructions. In other assays, IL-8(10ng/ml; Sigma-Aldrich) or anti-IL-6 neutralizing

antibody(10ng/ml; eBioscience, San Diego, CA,USA) were added into the culture system at the beginning of co-culture of PDLSCs and PMN. The cells were cultured in 0.2 ml RPMI-1640 at 37°C with 5% CO₂, and the percentage of apoptotic PMN was measured 24 hours later.

2.4 Transwell culture

Transwell chambers with a 0.4 µm pore size membrane (Costar, Cambridge, MA, USA) were used to separate the PMN from the PDLSCs [8]. PMN (5.0×10^4) were seeded in the upper chamber, and PDLSCs of equal number were placed in the bottom chamber. After 24 hours of co-culture, PMN were harvested, and the apoptotic percentage was measured.

2.5 Co-culture of PMN and IL-6

PMN and IL-6 of different concentrations (5ng/ml, 10ng/ml, 20ng/ml) were co-cultured for 24 hours. Then the percentage of apoptotic PMN was measured.

2.6 Statistical analysis

The results were collected and analyzed using SPSS 17.0 software (SPSS, IBM, Armonk, NY, USA). Statistical significance was assessed by two-tailed Student's t test or analysis of variance (ANOVA); a p-value less than 0.05 was considered statistically significant.

3 Results

When PDLSCs were co-cultured with resting PMN for 24 hours, the proliferation of PMN was elevated significantly. After the stimulation of PMN by IL-8, PDLSCs also could promote the proliferation of PMN, and there was no difference between the proliferation of resting PMN and stimulated PMN (Figure 1A).

PMN possess the property of spontaneous apoptosis. We then detect the apoptosis rate of PMN. Post-co-culture with PDLSCs, the apoptosis rate of PMN reduced significantly, whether IL-8 was added into the co-culture or not (Figure 1B).

Next we examined the mechanisms by which PDLSCs suppressed the PMN apoptosis. To examine whether cell-cell contact was required in this course, a Transwell

culture system was used to separate PMN from PDLSCs. The data showed that PMN apoptosis was equally suppressed by PDLSCs in both cell-cell contact culture and Transwell culture, suggesting that soluble factor(s) might be involved in the phenomenon (Figure 2A).

In addition, when anti-IL-6 neutralizing antibody was added into the co-culture of PDLSCs and PMN, the apoptosis of PMN increased markedly, comparable to that of the PMN cultured alone (Figure.2B).

After co-culture of PMN and IL-6 of different concentrations for 24 hours, IL-6 at the concentration of 10ng/ml and 20ng/ml could inhibit PMN apoptosis statistically, and there was no statistical difference between the groups of 10ng/ml and 20ng/ml. However, IL-6 at the concentration of 5ng/ml could not inhibit the apoptosis of PMN (Figure 3).

4 Discussion

Human immunity includes innate immunity and adaptive immunity, and cellular immunity and humoral immunity are two parts of adaptive immunity. Previous reports revealed that PDLSCs can suppress the cellular immunity and humoral immunity, including suppression of proliferation of T and B lymphocytes, and that transplantation of allogeneic PDLSCs did not induce immune responses [8-11]. However, the effect of PDLSCs on innate immunity is elusive. PMN is an important constituent of human innate immunity and the major inflammatory cells in the human body, playing important roles in maintaining homeostasis, withstanding the invasion of microbial pathogens, and improving the repair and regeneration of tissues [12].

PMN are a kind of terminally differentiating cell and normally short-living cells under normal physiological circumstances [13]. Our results showed that PDLSCs could improve the proliferation and reduce the apoptosis

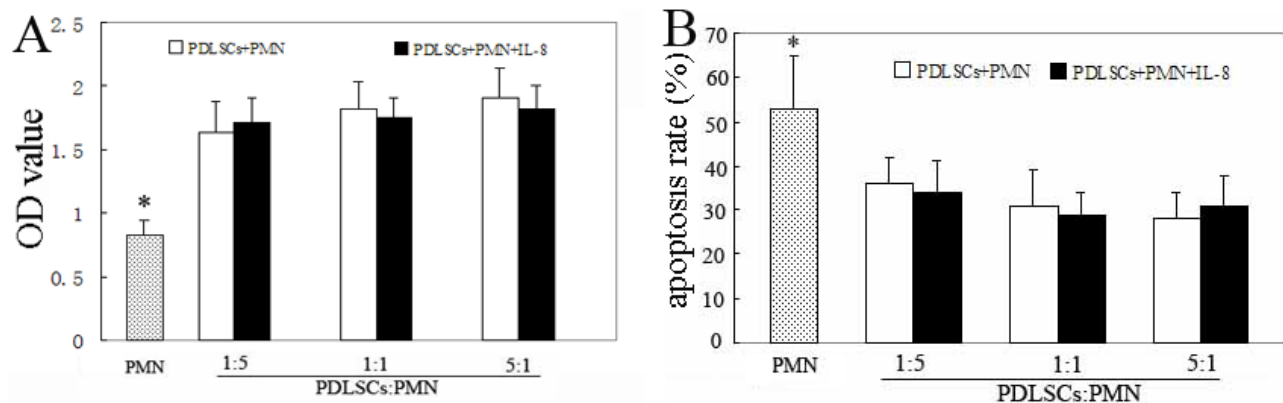


Figure 1: The effect of PDLSCs on the proliferation and apoptosis rate of PMN. **(A)** PDLSCs could elevate the proliferation of resting PMN and IL-8 stimulated PMN significantly. **(B)** The apoptosis rate of resting PMN and IL-8 stimulated PMN could be reduced by PDLSCs significantly. *: $P < 0.05$ vs other groups. Data are expressed as mean \pm SD of triplicates from six independent experiments.

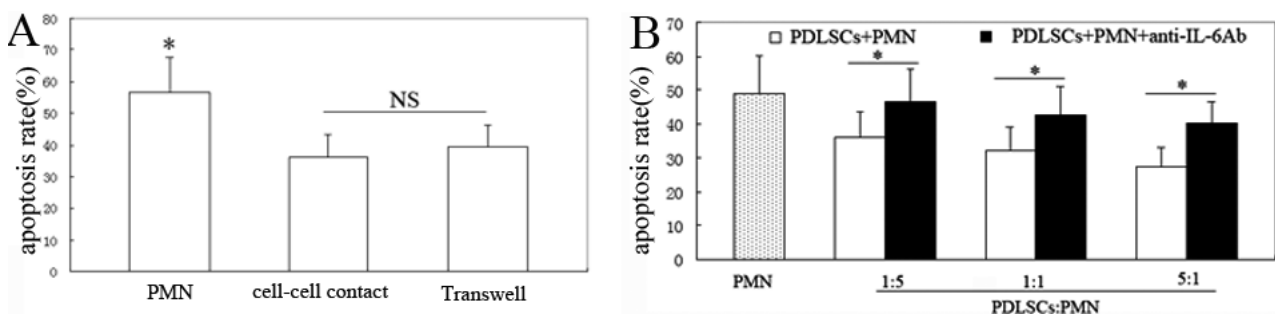


Figure 2: IL-6 is the critical factor in the PDLSCs-mediated reduction of PMN apoptosis. **(A)** PMN apoptosis was equally suppressed by PDLSCs in both cell-cell contact culture and Transwell culture. **(B)** The apoptosis of PMN increased markedly when anti-IL-6 neutralizing antibody was added into the co-culture of PDLSCs and PMN. *: $P < 0.05$ vs other groups. NS: not significant. Data are expressed as mean \pm SD of triplicates from six independent experiments.

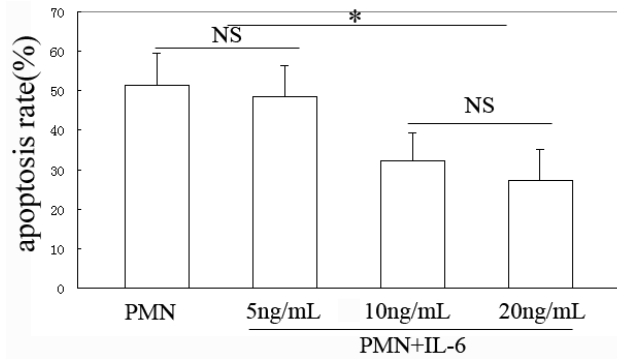


Figure 3: IL-6 at the concentration of 10ng/ml and 20ng/ml could inhibit PMN apoptosis statistically. However, IL-6 at the concentration of 5ng/ml could not inhibit the apoptosis of PMN. *: $P < 0.05$. NS: not significant. Data are expressed as mean \pm SD of triplicates from six independent experiments.

rate of resting PMN or IL-8 activated PMN, in accord with bone marrow derived MSCs [14], and which may markedly enhance the functions of PMN as the first line of defense against infectious pathogens in the inflammatory response [15].

To determine whether or not soluble factor is associated with PDLSCs-induced apoptosis suppression of PMN, we set up Transwell culture system to separate PDLSCs from PMN, and found that PDLSCs could reduce the PMN apoptosis in both cell-cell contact culture and Transwell culture, and there was no difference between the two culture conditions, implying that soluble factor takes part in the process.

IL-6 is a multi-functional cytokine and involved in various diseases including acute and chronic inflammation, and participates in the initiation of inflammation, which is capable of enhancing the proliferation and differentiation of T and B lymphocytes, and promotes the production and activation of PMN under inflammation [16, 17]. Previous studies showed that IL-6 was constitutively secreted by MSCs tested by cytokine mRNA expression array analyses, quantitatively by Multiplex and enzyme-linked immunosorbent assay analyses [18-21]. It was also found that IL-6 could prolong the survival time of PMN [22]. In addition, IL-6 was capable of inhibiting the apoptosis of PMN [23-26]. The proapoptotic protein Bax on PMN was down-regulated by IL-6, and the spontaneous activity of caspase-3 was also inhibited by IL-6 [23]. Furthermore, IL-6 was notably increased 24 hours postsurgery and at this postoperative concentration inhibited apoptosis of normal PMN [25]. In another study, IL-6 was regarded as the significant factor to inhibit exudative PMN apoptosis

which was markedly decreased on postoperative days [26]. Consistently, we observed that anti-IL-6 neutralizing antibody was able to restore the apoptosis of PMN when added into the co-culture of PDLSCs and PMN, showing that IL-6 plays a crucial role in the course.

This study showed that PDLSCs are able to reduce the apoptosis of PMN significantly, and IL-6 is involved.

Acknowledgements: This work was supported by grants from the National Natural Science Foundation of China (No.81570945 to G.D.), and the Natural Science Foundation of Shandong Province (No. ZR2015HL055 to G.D.).

Conflict of interest statement: Authors state no conflict of interest.

References

- [1] Pittenger M.F., Mackay A.M., Beck S.C., Jaiswal R.K., Douglas R., Mosca J.D., et al., Multilineage potential of adult human mesenchymal stem cells, *Science*, 1999, 284, 143-147
- [2] Seo B.M., Miura M., Gronthos S., Bartold P.M., Batouli S., Brahimi J., et al., Multipotent postnatal stem cells from human periodontal ligament, *The Lancet*, 2004, 364, 149-155
- [3] Seo B.M., Miura M., Sonoyama W., Coppe C., Stanyon R., Shi S., Recovery of stem cells from cryopreserved periodontal ligament, *J. Dent. Res.*, 2005, 84, 907-912
- [4] Sonoyama W., Liu Y., Fang D., Yamaza T., Seo B.M., Zhang C., et al., Mesenchymal stem cell-mediated functional tooth regeneration in swine, *PLoS One*, 2006, 1, e79
- [5] Liu Y., Wang L., Kikui T., Akiyama K., Chen C., Xu X., et al., Mesenchymal stem cell-based tissue regeneration is governed by recipient T lymphocytes via IFN- γ and TNF- α , *Nat. Med.*, 2011, 17, 1594-1601
- [6] Tang R., Wei F., Wei L., Wang S., Ding G., Osteogenic differentiated periodontal ligament stem cells maintain their immunomodulatory capacity, *J. Tissue Eng. Regen. Med.*, 2014, 8, 226-232
- [7] Brandau S., Jakob M., Hemeda H., Bruderek K., Janeschik S., Bootz F., et al., Tissue-resident mesenchymal stem cells attract peripheral blood neutrophils and enhance their inflammatory activity in response to microbial challenge, *J. Leukoc. Biol.*, 2010, 88, 1005-1015
- [8] Ding G., Liu Y., Wang W., Wei F., Liu D., Fan Z., et al., Allogeneic periodontal ligament stem cell therapy for periodontitis in swine, *Stem Cells*, 2010, 28, 1829-1838
- [9] Liu O., Xu J., Ding G., Liu D., Fan Z., Zhang C., et al., Periodontal ligament stem cells regulate B lymphocyte function via programmed cell death protein, *Stem Cells*, 2013, 31, 1371-1382
- [10] Liu D., Xu J., Liu O., Fan Z., Liu Y., Wang F., et al., Mesenchymal stem cells derived from inflamed periodontal ligaments exhibit impaired immunomodulation, *J. Clin. Periodontol.*, 2012, 39, 1174-1182

- [11] Wada N., Menicanin D., Shi S., Bartold P.M., Gronthos S., Immunomodulatory properties of human periodontal ligament stem cells, *J. Cell Physiol.*, 2009, 219, 667-676
- [12] Witko-Sarsat V., Rieu P., Descamps-Latscha B., Lesavre P., Halbwachs-Mecarelli L., Neutrophils: Molecules, functions and pathophysiological aspects, *Lab. Invest.*, 2000, 80, 617-653
- [13] Chou R.C., Kim N.D., Sadik C.D., Seung E., Lan Y., Byrne M.H., et al., Lipid-cytokine-chemokine cascade drives neutrophil recruitment in a murine model of inflammatory arthritis, *Immunity*, 2010, 33, 266-278
- [14] Raffaghello L., Bianchi G., Bertolotto M., Montecucco F., Busca A., Dallegri F., et al., Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche, *Stem Cells*, 2008, 26, 151-162
- [15] Cassatella M.A., Mosna F., Micheletti A., Lisi V., Tamassia N., Cont C., et al., Toll-like receptor-3-activated human mesenchymal stromal cells significantly prolong the survival and function of neutrophils, *Stem Cells*, 2011, 29, 1001-1011
- [16] Kishimoto T., IL-6: from its discovery to clinical applications, *Int. Immunol.*, 2010, 22, 347-352
- [17] Scheller J., Chalaris A., Schmidt-Arras D., Rose-John S., The pro- and anti-inflammatory properties of the cytokine interleukin-6, *Biochim. Biophys. Acta.*, 2011, 1813, 878-888
- [18] Kim D.H., Yoo K.H., Choi K.S., Choi J., Choi S.Y., Yang S.E., et al., Gene expression profile of cytokine and growth factor during differentiation of bone marrow-derived mesenchymal stem cell, *Cytokine*, 2005, 31, 119-126
- [19] Kögler G., Radke T.F., Lefort A., Sensken S., Fischer J., Sorg R.V., et al., Cytokine production and hematopoiesis supporting activity of cord blood-derived unrestricted somatic stem cells, *Exp. Hematol.*, 2005, 33, 573-583
- [20] Gunn W.G., Conley A., Deininger L., Olson S.D., Prockop D.J., Gregory C.A., A crosstalk between myeloma cells and marrow stromal cells stimulates production of DKK1 and interleukin-6: a potential role in the development of lytic bone disease and tumor progression in multiple myeloma, *Stem Cells*, 2006, 24, 986-991
- [21] Wagner W., Roderburg C., Wein F., Diehlmann A., Frankhauser M., Schubert R., et al., Molecular and secretory profiles of human mesenchymal stromal cells and their abilities to maintain primitive hematopoietic progenitors, *Stem Cells*, 2007, 25, 2638-2647
- [22] Daffern P.J., Jagels M.A., Hugli T.E., Multiple epithelial cell-derived factors enhance neutrophil survival. Regulation by glucocorticoids and tumor necrosis factor-alpha, *Am. J. Respir. Cell Mol. Biol.*, 1999, 21, 259-267
- [23] Ottonello L., Frumento G., Arduino N., Bertolotto M., Dapino P., Mancini M., et al., Differential regulation of spontaneous and immune complex-induced neutrophil apoptosis by proinflammatory cytokines. Role of oxidants, Bax and caspase-3, *J. Leukoc. Biol.*, 2002, 72, 125-132
- [24] Asensi V., Valle E., Meana A., Fierer J., Celada A., Alvarez V., et al., In vivo interleukin-6 protects neutrophils from apoptosis in osteomyelitis, *Infect. Immun.*, 2004, 72, 3823-3828
- [25] Fanning N.F., Porter J., Shorten G.D., Kirwan W.O., Bouchier-Hayes D., Cotter T.G., et al., Inhibition of neutrophil apoptosis after elective surgery, *Surgery*, 1999, 126, 527-534
- [26] Matsuda T., Saito H., Fukatsu K., Han I., Inoue T., Furukawa S., et al., Cytokine-modulated inhibition of neutrophil apoptosis at local site augments exudative neutrophil functions and reflects inflammatory response after surgery, *Surgery*, 2001, 129, 76-85

Abbreviations

MSCs: mesenchymal stem cells

PDLSCs: periodontal ligament stem cells

PMN: polymorphonuclear neutrophils