Effect of TNF-α on the proliferation and osteogenesis of human periodontal mesenchymal stem cells

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Abstract. The aim of the present study was to investigate the effect of tumor necrosis factor-α (TNF-α) on the proliferation and osteogenesis of human periodontal mesenchymal stem cells (hPDLSCs). Antigen expression in hPDLSCs was detected by flow cytometry. hPDLSCs were divided into four groups: A control group with no TNF-α treatment, and three experimental groups treated with 0.1, 1 and 10 ng/ml TNF-α, respectively. The effect of TNF-α on proliferation of hPDLSCs in vitro was detected using a Cell Counting Kit-8 assay. Differentiation into an osteogenic lineage was detected by alkaline phosphatase sand alizarin red staining, and the mRNA and protein expression levels of runt-related transcription factor 2 (Runx2), osteocalcin (OCN) and type I collagen (Col-I) were detected using reverse transcription-quantitative PCR and western blot respectively. Following treatment with 10 ng/ml TNF-α, proliferation was significantly increased compared with an untreated control group (P<0.01). Additionally, there was a significant inhibition of alkaline phosphatase enzyme activity, alizarin red mineralization node size, and in the gene and protein expression levels of osteogenic differentiation markers, including Runx2, OCN and COL-1 (all, P<0.05). Taken together, the results indicated that treatment with 10 ng/ml TNF-α promoted the proliferation of hPDLSCs in vitro and inhibited osteogenic differentiation of hPDLSCs, providing an experimental basis for regulation of hPDLSC-mediated periodontal tissue regeneration.

Introduction

Periodontal disease is one of the most common oral diseases in adults in China (1). It causes periodontal ligament, cementum and alveolar bone desctruction, gingiva, tooth loosening and may leads to the loss of teeth. Previous studies have demonstrated that chronic periodontitis can aggravate or directly affect cardiovascular disease, diabetes, rheumatism, digestive system disease, eye disease and immune function in the body (2-4). The existing periodontal treatment methods include periodontal basic treatment, flap curettage, root planing, application of growth factors (5,6), natural material grafts and guided tissue regeneration (GTR) (7), which can only partially regenerate periodontal tissue. Additionally, the curative effect is not stable. Therefore, it is necessary to elucidate novel and more effective periodontal treatment methods in the clinic (8). Human periodontal mesenchymal stem cells (hPDLSCs) derived from periodontal ligament are a suitable source of stem cells for periodontal tissue regeneration due to the ease of acquisition, simple means of culturing and isolation, multilineage potential and low immunogenicity (9). The release of inflammatory mediators, such as TNF-α, not only accelerates the progression of periodontitis, but also affects the regeneration of hPDLSCs (10). It was reported that TNF-α inhibits the osteogenic differentiation of mesenchymal stem cells (MSCs), such as bone marrow stem cells (11,12). However, studies on the effect of TNF-α on the osteogenic ability of hPDLSCs are relatively rare and have provided contradictory results (13,14). Therefore, the aim of the present study was to determine the effect of TNF-α on the proliferation and osteogenic ability of hPDLSCs, providing an experimental basis...
for exploring antagonistic targets of TNF-α and improving the understanding of regeneration and repair of hPDLSCs in periodontitis.

Materials and methods

Isolation, purification and culture of hPDLSCs. The acquisition and cultivation of hPDLSCs was approved by the Ethics Committee of the Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China) and informed consent was provided by the patients in writing. The patients agreed to the use of their teeth for scientific research. A total of 10 teeth were extracted during routine dental care in the Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine were collected from 6 female and 4 male patients. Teeth with no periodontal abnormalities, no inflammation and no caries were obtained from July 2018 to July 2019. The age of the patients ranged from 18-25 years, with a mean age of 20.7±3 years. Premolars were washed with PBS three times, and the periodontal ligament tissue from the middle and lower part of the root was scraped, shredded, centrifuged (400 x g for 5 min at 37°C), digested with trypsin and placed in 10% FBS α-MEM culture medium (HyClone; Cytiva) containing penicillin-streptomycin, and cultured at 37°C with 5% CO₂ in a humidified incubator. The culture medium was changed every 3 days. After the cells reached ~80% confluence, they were digested with trypsin and sub-cultured at a ratio of 1:3.

Identification of cell surface antigens. Cells were cultured in vitro for three passages, after which they were digested using trypsin-EDTA, and centrifuged at 400 x g for 5 min at 37°C, and the supernatant was removed. The cell suspension was prepared by trypsin digestion and the cell density was adjusted to 1x10⁶/ml. Subsequently, the cell suspension was divided into five 1.5 ml Eppendorf tubes containing the following antibodies: 5 µl mouse anti-human STOR-1 (1:1,000; cat. no. ab92395; Abcam), mouse anti-human FITC-CD 90 (1:500; cat. no. BD-561969; BD Pharmingen; BD Biosciences) mouse anti-human FITC-CD34 (1:500; cat. no. BD-555821; BD Pharmingen; BD Biosciences), mouse anti-human FITC-CD45 (1:500; cat. no. BD-555482; BD Pharmingen; BD Biosciences), and FITC labeled mouse anti-human IgG1 (1:1,000; cat. no. ab99773; Abcam). After incubation for 1 h at room temperature, the expression of cell surface markers was detected by flow cytometry (Attune NxT; Thermo Fisher Scientific, Inc.) using Invitrogen Attune NxT software version 4.2 (Invitrogen; Thermo Fisher Scientific, Inc.).

Effect of TNF-α on the proliferation of hPDLSCs. A Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) assay was used to determine the effect of different concentrations of TNF-α (PeproTech, Inc.) on the proliferation of hPDLSCs. Cell cultures were divided into four groups. Osteogenic induction solution (α-MEM containing 10 mmol/l dexamethasone, 10 mmol/l insulin, 0.2 mmol/l indomethacin and 0.5 mmol/l 3-isobutyl-1-methylxanthine) was added to the control group without TNF-α. The other three experimental groups were treated with osteogenic induction medium containing 0.1 ng/ml TNF-α, 1 ng/ml TNF-α and 10 ng/ml TNF-α, respectively.

The original culture medium was discarded, cells were washed with PBS three times, digested with trypsin and centrifuged at 400 x g for 5 min at 37°C. The cells were cultured in 100 µl medium at a density of 2x10⁴ cells/well in a 96-well plate and cultured at 37°C for 24 h. The culture medium was changed every 3 days and cultured continuously for 7 days. On day 7, 10 µl of CCK-8 solution was added to each well, and cells were further incubated for 2 h and gently mixed on a shaker for 5 min to ensure uniform reagent distribution. The optical density at 450 nm was measured using a microplate reader. The growth curve was drawn with observation time on the x-axis and the OD value on the y-axis, to analyze and compare cell proliferation.

Effect of TNF-α on osteogenic differentiation of hPDLSCs. Quantitative alkaline phosphatase (ALP) enzyme ELISA kit (cat. no. YM-S2942) and alizarin red staining were used to detect the effect of different concentrations of TNF-α on the osteogenic induction of hPDLSCs. The osteogenic induction solution without TNF-α was added to the control group and the osteogenic induction solution containing 10 ng/ml TNF-α was added to the experimental group. After 7 days of induction culture at 37°C, there was intact cell morphology of spindle or triangular shape in the 96-well plate when observed under a light microscope. The cells were washed using 10%PBS. A total of 50 µl from each well was collected and transferred to another 96-well plate at a density of 1x10⁴ cells/ml. Buffer, substrate and chromogenic solution (provided by the ELISA kit) were added in turn, and the ALP activity of each well was measured at 520 nm.

After osteogenic induction and culture for 21 days as aforementioned, the culture medium was discarded, the cells were fixed with 4% paraformaldehyde for 10 min at 4°C, and stained with alizarin red S solution for 15 min at 37°C. The formation of mineralized nodules was observed under an inverted phase contrast microscope (Olympus Corporation) and imaged.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following spectrophotometric quantification using a NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.), 1 µg total RNA in a final volume of 20 µl was used for RT with a PrimeScript RT Reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. Aliquots of cDNA were used for mRNA quantification by qPCR using a LightCycler 96 Real-time Quantitative PCR Detection system (Roche Applied Science). The thermocycling conditions were as follows: 35 cycles of denaturation at 94°C for 30 sec, annealing at 37°C for 30 sec, and extension at 72°C for 1 min. The reaction system (25 µl) contained the cDNA, forward and reverse primers, and SYBR-Green PCR MasterMix (Roche Applied Science). Data were analyzed using β-actin gene expression as the internal standard, and the 2⁻ΔΔCt method was used for quantification (15). The sequences of the primers were based on a previous study (16): TNF-α forward, 5'-CTCCCTAGCAAAACACTGACG-3' and reverse, 5'-CTT GGCAGATTGACCTCAGC-3'; β-actin forward, 5'-CCACAC CGGCCCACTGTTG-3' and reverse, 5'-CCCCATCCCACTCATCAC-3'; Runx2 forward, 5'-TGAAATAGGACGTCAGG-3' and reverse, 5'-GCTGTTGCTCTTGTTG-3'; CCL3 forward, 5'-CAATCCAGGGTTCTACACG-3' and reverse, 5'-GACACGCTCGGTTGCT-3'.
ACA AAA-3' and reverse, 5'-CAG TAG CAA ACC GAA ACA CT-3'; Col-I forward, 5'-AGT GGT TTG GAT GGT GCC AA-3' and reverse, 5'-GCA CCA TCA TTT CCA CGA GC-3'; OCN forward, 5'-ATG AGA GCC CTC ACA CTC CT-3' and reverse, 5'-CTTGAGACAAAGGCTGCAC-3'.

Western blotting. The effect of TNF-α on the expression of osteogenesis-related marker proteins was detected by western blotting. Total proteins were extracted, and the concentration of each protein sample was determined. Cells were harvested and lysed on ice in RIPA Lysis Buffer (Beyotime Institute of Biotechnology). A bicinchoninic acid assay (Thermo Fisher Scientific, Inc.) was performed to measure the concentration of protein. Equal quantities of protein (50 µg) were loaded on an SDS gel, resolved using SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked in 5% skimmed milk for 2 h. Subsequently the membranes were incubated with primary antibodies against runt-related transcription factor 2 (Runx2), osteocalcin (OCN) and collagen type Iα1 (COL-I) and β-actin (all 1:1,000) overnight at 4˚C. Subsequently, the membranes were washed three times with PBS containing 0.05% Tween-20 and incubated with a horse-radish peroxidase-conjugated secondary antibody (1:5,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. The membranes were washed again, and target bands and detected using an enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.) and analyzed using ImageJ software v1.8.0 (National Institutes of Health). β-actin was used as the internal control. The experiments were repeated three times.

Statistical analysis. Data were analyzed using SPSS version 13 (SPSS, Inc.). Data are presented as the mean ± standard deviation of three repeats. Differences between two groups were compared using a paired Student's t-test. The significance of differences among more than two groups was assessed using the one-way ANOVA followed by Tukey-Kramer multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

Results

Isolation of hPDLSCs. When the primary hPDLSCs cells were cultured for 3 days, a few cells could be obtained from the tissue block, with an oval and polygonal shape, and of different sizes. The number of the cells began to increase after 5 days, with cells exhibiting a long spindle or polygonal morphology. After 6-8 days of cultivation, occurring prior to osteogenic induction, the number of the cells markedly increased, with the majority of cells exhibiting a long spindle, short spindle or polygonal morphology (Fig. 1A). When the cell confluence rate reached ~80%, they were sub-cultured by selective digestion. The purity and number of the cells after three passages was higher compared with the primary cells, and the growth was considered to be healthy, with no visible abnormalities. The size of the cells was even, they were arranged closely, and predominantly exhibited a long spindle and fiber-like morphology (Fig. 1B).

Evaluation of surface antigens on the hPDLSCs. The expression of surface antigen molecules on hPDLSCs was assessed using flow cytometry (Fig. 2). The results showed that amongst the established markers of MSCs assessed, the proportion of cells expressing STOR-1 was 99.95%, and those expressing CD90 accounted for 99.75% of the cells. Amongst the markers of blood cell growth, the proportion of cells expressing CD34 was 1.42 and 0.37% cells were positive for CD45. The results of flow cytometry indicated that hPDLSCs cells were successfully isolated and extracted.

Effect of TNF-α on the osteogenic ability of hPDLSCs. Induction of osteogenesis begun on day 7, and the detection of ALP activity showed that in both groups (treated and untreated with TNF-α), ALP activity gradually increased with cultivation time when
compared with the control group, and 10 ng/ml TNF-α significantly reduced ALP activity (Fig. 4). After 21 days of osteogenesis induction, alizarin red staining showed that compared with the control group, the number and size of red mineralized nodules decreased in the test group (10 ng/ml TNF-α), and the staining was lighter (Fig. 5).

**Effect of TNF-α on mRNA and protein expression of osteogenesis-associated genes.** Compared with the control group, 10 ng/ml TNF-α significantly reduced Runx2, OCN and COL-I mRNA expression levels (P<0.001 Fig. 6). Similarly, 10 ng/ml TNF-α significantly reduced Runx2, OCN and COL-I protein expression levels compared with the control group (all P<0.001; Fig. 7).
Discussion

Periodontitis can result in swelling of the gums, pain, alveolar bone destruction, occlusal function weakness, tooth loss and/or complete loss of occlusal function, and may thus severely affect a patient’s quality of life and health (17). MSCs exhibit self-replication and multi-lineage differentiation potential, and are considered a type of adult stem cells that are widely present in human tissues. Under specific conditions, MSCs can differentiate into bone, cartilage, fat, muscle and nerve tissue (18). hPDLSCs are a type of MSCs widely used and studied in periodontal regeneration engineering, and can survive, proliferate and differentiate into cementum, alveolar bone and periodontal ligament-like tissue, and may thus serve as a source of stem cells for periodontal tissue regeneration (19,20). Previous clinical studies have shown that periodontal ligament stem cells were safe and effective in the treatment of periodontal diseases (21).

Enzyme digestion, tissue culture, or a combination of both are commonly used in studies involving acquisition, purification and culture of PDLSCs (22). In the present study, the combined method of enzyme digestion and tissue culture was used to isolate and culture hPDLSCs. This approach was superior to the use of enzyme digestion alone, as it enabled extraction of a greater number of cells and avoided the difficulties in having to adjust the extent of digestion (22). The method used in the current study was also superior to the use of tissue culture alone, which often results in incomplete digestion and low cell survival rates (23). As a result, primary hPDLSCs were obtained and sub-cultured successfully in the present study. At present, to the best of our knowledge, there are no specific identification methods for hPDLSCs. Characteristics of MSCs include high expression of stem cell growth-related proteins, such as antigen molecules including STOR-1, CD105,
CD90 and CD73; and almost no expression of blood cell growth-related proteins, such as CD34, CD45 and HLA-DR, and these were used to validate whether the cells obtained in the present study were indeed hPDLSCs. After successful sub-culturing of hPDLSCs, the cells were shown to be of a high level of purity, with a high proliferation capacity, with no visible abnormalities, uniform cell size and compact arrangement, exhibiting a long fusiform and fibrous morphology. Flow cytometry was used to confirm the expression of surface antigen molecules, and it was found that the stem cell-related proteins STOR-1 and CD105 were abundantly expressed, whereas expression of the blood cell growth-related proteins CD34 and CD45 were detected at low levels. Together these results provided confidence that the cells extracted and isolated were hPDLSCs.

Periodontitis is a ubiquitous periodontal tissue immune inflammatory disease caused by bacteria (24). The regenerative ability of hPDLSCs is readily affected by the presence of inflammatory mediators such as TNF-α, IL-6, IL-11 and IL-17 in the periodontal environment (25,26). Among these, TNF-α is an important member of the TNF family, showing its effect primarily through the NF-κB or MAPK signaling pathways. It is also the primary inflammatory factor regulating tissue destruction in periodontitis, and is involved in several different inflammatory reactions (27).

In the present study, the effects of different concentrations of TNF-α on the proliferation of hPDLSCs in vitro were compared, and it was found that only 10 ng/ml TNF-α could promote the proliferation of hPDLSCs in vitro. It has been previously shown that 10 ng/ml TNF-α significantly promoted the proliferation of dental pulp stem cells and hPDLSCs (28). In the present study, the effect of TNF-α on cell proliferation was detected using a CCK-8 assay, and the results were consistent with the previous study, where 10 ng/ml TNF-α promoted the proliferation of hPDLSCs (28). However, it has also been reported that 10 ng/ml TNF-α can significantly induce apoptosis in dental pulp stem cells and hPDLSCs (29). The effect of TNF-α on the proliferation of hPDLSCs in vitro is complex and diverse, and this may be related to the source and method of acquisition of hPDLSCs, culture conditions and detection methods.

ALP, OCN, COL-I and Runx2 are important factors associated with osteogenesis, and serve important roles in the formation and development of osteocytes, and are thus used as an index to assess the osteogenic induction and differentiation ability of MSCs. Previous studies have shown that TNF-α can inhibit osteoblast bone formation, promote bone resorption, inhibit ALP activity and the mRNA levels of ALP, Runx2 and osterix (30,31). In the present study, 10 ng/ml TNF-α was used to inhibit the osteogenic differentiation of normal periodontal ligament stem cells. The decrease in ALP activity and alizarin red staining, and the decreased mRNA and protein expression levels of Runx2, COL-I and OCN showed that 10 ng/ml TNF-α could inhibit the osteogenic differentiation of hPDLSCs.

In conclusion, the present study indicated that 10 ng/ml TNF-α significantly promoted the proliferation of hPDLSCs and inhibited osteogenic differentiation. These results highlight novel potential means of regulating regeneration of hPDLSCs through the use of specific concentrations of inflammatory factors such as TNF-α for the prevention and treatment of periodontitis.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

QJ and LZ conceived and designed the study. QJ and YC performed the experiments. QJ, YC, YW and CL analyzed the data. YC and CL wrote the manuscript. All authors read and approved the final manuscript. QJ and YC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocols used in the present study were approved by the Ethics Committee of the Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China) and informed consent was provided by the patients. The ethics approval reference number is [2018] 376. The patients agreed to the use of their teeth for scientific research.

Patient consent for publication

All patients provided written informed consent for the publication of their anonymized clinical data.

Competing interests

The authors declare that they have no competing interests.

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