Concentrated growth factor exudate enhances the proliferation of human periodontal ligament cells in the presence of TNF-α

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Abstract. The purpose of this study was to evaluate the effects of concentrated growth factor exudate (CGFe) on human periodontal ligament cells (hPDLCs) stimulated by tumor necrosis factor (TNF)-α. From the peripheral blood of healthy donors, CGFe was prepared according to the Sacco protocol after 7 days of incubation. The hPDLCs were cultured by a tissue explant method and identified with anti-vimentin and anti-cytokeratin antibodies. Cells were subjected to four different treatments: i) Control; ii) TNF-α (10 ng/ml); iii) CGFe (concentration 50%); and iv) CGFe+TNF-α. The proliferation of hPDLCs was measured with Cell Counting Kit-8 assays. Osteogenic differentiation and mineralization were determined by Alizarin Red S staining, alkaline phosphatase activity, western blotting and reverse transcription-quantitative polymerase chain reaction. CGFe enhanced cell proliferation and upregulated ALP activity, the mineralization level, and osteogenic-associated osteocalcin, runt-related transcription factor 2 and Osterix gene expression in hPDLCs under inflammatory conditions induced by TNF-α. The present study demonstrated that CGFe enhanced hPDLC proliferation and osteogenic differentiation in the presence of TNF-α-induced inflammation. As CGFe can be obtained from the venous blood of patients, it generates no immune reaction. Thus, it is more economical and beneficial to use CGFe in clinical periodontal regeneration practice than synthetic growth factors.

Introduction

Periodontitis and chronic apical periodontitis often cause periodontal tissue destruction and severe alveolar bone defects (1). In periodontally compromised teeth, when partial or full socket wall destruction is evident, connective tissue will grow into the extraction site and lead to a deficient ridge (1). Periodontitis is widely treated with instant implant technology in clinical practice (2). However, periodontal tissue destruction and alveolar bone defects significantly weaken the stability and shorten the service life of dental implants (2). To improve the success rate of dental implants, concentrated growth factor (CGF) can be used to promote periodontal tissue regeneration and osteogenic differentiation (3). CGF is a gel-like substance, obtained by centrifugation of venous blood, which is rich in growth factors and fibrin (4-8), and CGF combined with bone graft material promotes immediate periodontal tissue regeneration and osteogenic differentiation (8,9).

Previous studies have reported that platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) in the first and second phases promote tissue regeneration, inhibit infection, regulate inflammation and reduce postoperative reactions (9-14). CGF is a third-phase plasma extract, consisting of multiple growth factors (4-8). Due to the unique structure of CGF fibrin, it can replace the diaphragm used in guiding bone regeneration to a certain extent (8,9,15). CGF has been widely studied in the fields of oral implantation, extraction site preservation, jaw cyst treatment, and fracture healing promotion (4,8,16,17).

CGF exudate (CGFe) is extracted from CGF and used in research labs to study the effects of CGF. Previous studies have shown that CGFe significantly shortens the time for osteogenesis in the operational area and distinctly improves bone formation quality (8,9). CGF has been demonstrated to stimulate the proliferation of human periodontal ligament fibroblasts (PDLFs) (17). However, these previous studies were performed without the influence of inflammatory factors, and therefore did not sufficiently reflect the clinical environment. Particularly, the effect of CGFe on enhancing the proliferation of hPDLCs in the presence of tumor necrosis factor (TNF)-α-induced inflammation has not yet been investigated, to the best of our knowledge.
TNF-α is a pro-inflammatory cytokine that is a critical pathological factor for the development of apical inflammation, which significantly inhibits osteogenic differentiation (16,18-21). As TNF-α-induced inflammation is commonly encountered in clinical practice (16,20), it is of great clinical interest to evaluate the effects of CGFe in the presence of TNF-α.

In the present study, the effects of CGFe on hPDLC proliferation and osteogenic differentiation were investigated in an inflammatory environment, stimulated by TNF-α. Specifically, the effects of CGFe on alkaline phosphatase (ALP) activity, mineralization, and osteocalcin (OCN), runt-related transcription factor 2 (RUNX2), and Osterix (OSX) gene expression in hPDLCs under TNF-α-induced inflammatory conditions were examined.

Materials and methods

Preparation of CGFe. The present study was approved by the Ethics Committee of the Jilin University Health Science Center (Changchun, China). In accordance with this committee, CGFe was obtained from three healthy male donors who had visited the outpatient clinic at the Health Center of Jilin University (Changchun, China) from August 2017 to March 2018. They were non-smokers and non-drinkers aged from 22 to 30 years old, and their informed consent was obtained. The experiments described below were carried out under similar conditions and procedures as the previous study (11).

Venous blood samples from the donors were used to produce human CGF according to a previously described protocol (22). In brief, blood samples were centrifuged at 750 x g for 12 min at 4°C. Between the acellular plasma and red blood cells (RBCs), a white CGF clot formed. The CGF clot was held with sterile forceps, separated from the RBCs using scissors, placed on the grid of an endo box and compressed by the endo box cover. After 1 min of applied pressure, the CGF clot was converted into CGF membrane, and the CGF exudate (CGFe) was collected in the tray of the endo box.

The CGFe was centrifuged at 500 x g for 5 min at 4°C to remove RBCs. Then, the CGFe was precipitated and filtered using a 0.22 µm sterile syringe filter unit (Merck KGaA, Darmstadt, Germany). The pooled CGFe samples were stored at -80°C prior to use. The original concentration of CGFe was defined as 100%, and a 50% concentration of CGFe was obtained by dilution with Minimal Essential Medium-α (α-MEM; Gibco; Thermo Fisher Scientific, Inc.) and 22.52 mg/ml glycerine in PBS + 0.1% Tween-20 for 30 min at room temperature.

Next, cells were incubated with anti-vimentin (1:100; cat. no. ab24252; Abcam, Cambridge, MA, USA) and anti-cytokeratin (1:200; cat. no. AM06387SU-N; OriGene Technologies, Inc., Beijing, China) primary antibodies overnight at 4°C. The SP immunohistochemistry assay kit (cat. no. SP9001; ZSGB-BIO; OriGene Technologies, Inc., Beijing, China) was used for immunocytocchemical staining according to the manufacturer's instructions, and a 3,3’-diaminobenzidine was used to stain positive cells. An inverted microscope (IX73; Olympus Corporation, Tokyo, Japan) was used to view the cells at magnifications of x20 or x40.

Cell Counting Kit (CCK)-8 assay. The objective of this experiment was to examine the effect of CGFe on proliferation of hPDLCs in vitro. CGFe was obtained according to a previous protocol (16). The CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) was used to determine the effects of CGFe on hPDLC proliferation.

hPDLCs (2x10^4/well) were seeded into a 96-well plates containing complete medium (α-MEM supplemented with 10% FBS, 1% streptomycin and penicillin) with 10% FBS and incubated for 24 h at 37°C. Then, hPDLCs were exposed to CGFe (concentration 50%), TNF-α (10 ng/ml), or CGFe+TNF-α for 24, 48 or 72 h at 37°C, respectively. There was one additional control group with no CGFe or TNF-α treatment (complete medium with 10% FBS only). Cell proliferation was determined using the CCK-8 kit at the specified time-points. Kit reagent (10 µl) was added to the culture medium in each well. After a 90 min incubation at 37°C, absorbance at 450 nm was detected using an automatic microplate reader (Infinite 200 PRO; Tecan Group Ltd., Mannedorf, Switzerland). A well containing complete medium and CCK-8 solution without seeding cells was used as a further control. The assay was performed in duplicate, and the experiments were repeated six times under the same conditions.

ALP activity assay. hPDLCs (500 µl; 1x10^3/well) were seeded into 24-well plates and incubated for 24 h at 37°C. Next,
the cells were exposed to CGFe, TNF-α or CGFe+TNF-α for 7 or 14 days. At the given time-points, cells were lysed with 0.1% Triton X-100, and the lysates were centrifuged at 8000 x g for 10 min at 4°C. The supernatant (50 µl/well) was added to 96-well plates, and ALP activity was examined with a ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols. The optical density (OD) values were read at 520 nm with an automatic microplate reader (Infinite 200 PRO; Tecan Group, Ltd.).

**Osteogenic differentiation induction and Alizarin Red S staining.** hPDLCs (500 µl; 1x10⁵/well) were seeded into 24-well plates in standard medium (α-MEM supplemented with 10% FBS, 1% streptomycin and penicillin) until 60-70% confluence was reached. The medium was then replaced with four different media: i) Mineral induction medium (MM; α-MEM medium containing 10% FBS, 50 mg/ml ascorbic acid, 10 mM β-glycerophosphate and 10 M dexamethasone); ii) MM with CGFe (concentration 50%); iii) MM with TNF-α (10 ng/ml); or iv) MM with CGFe+TNF-α (10 ng/ml). The medium was replaced every 3 days.

Alizarin Red staining was used to detect and quantify the formation of mineralized nodules after 21 days. This assay was performed according to a previously described protocol, with minor modifications (9,23). Specifically, the hPDLCs were fixed with 95% ethanol for 15 min at room temperature, washed twice with dH₂O and stained with 0.1% Alizarin Red S solution (pH 4.1) for 20 min at room temperature. hPDLCs were observed under an inverted phase-contrast microscope (IX73; Olympus Corporation, Tokyo, Japan; magnification, x20 or x40). Next, the hPDLCs were washed three times with dH₂O. To semi-quantify the content of mineralized matrix nodules generated from the hPDLCs, 100 mM cetyl pyridinium chloride was added to the 24-well plates to dissolve and release the calcium-combined Alizarin Red S into solution. OD values were read at 570 nm, which represented the relative quantity of mineralization nodules.

**Western blotting.** Cell lysates were prepared in radioimmunoprecipitation assay buffer (150 mM NaCl, 0.1% SDS, 1 mM PMSF, 10 mM Tris-Cl, pH 7.4, 1% sodium deoxycholate, 1% Triton X-100). The cells were treated with four different media (control, CGFe, TNF-α, or CGFe+TNF-α) for 14 days. The cell lysates were incubated for 30 min on ice, then clarified by centrifugation at 6,000 x g for 10 min, and then centrifuged at 6,000 x g for 10 min at 4°C. Protein concentration was determined with a bicinchoninic acid determination (pH 4.1) for 20 min at room temperature. hPDLCs were fixed with 95% ethanol for 15 min at room temperature, washed twice with dH₂O and stained with 0.1% Alizarin Red S solution (pH 4.1) for 20 min at room temperature. hPDLCs were observed under an inverted phase-contrast microscope (IX73; Olympus Corporation, Tokyo, Japan; magnification, x20 or x40). Next, the hPDLCs were washed three times with dH₂O. To semi-quantify the content of mineralized matrix nodules generated from the hPDLCs, 100 mM cetyl pyridinium chloride was added to the 24-well plates to dissolve and release the calcium-combined Alizarin Red S into solution. OD values were read at 570 nm, which represented the relative quantity of mineralization nodules.

**Statistical analysis.** hPDLC proliferation and ALP activity assays were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Western blotting and Alizarin Red staining assay data were analyzed using two-way ANOVA, followed by Bonferroni's post-hoc comparisons test for independent samples. SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analysis. P<0.05 was considered to indicate a statistically significant difference. All data were expressed as the mean ± standard deviation, and experiments were performed in triplicate.

**Results**

**Characterization of hPDLCs.** hPDLCs grew out from the tissue explant after 7 and 10 days of culture (Fig. 1A). Spindle shapes were observed, and a number of cells were distributed in a circinate pattern with rapid proliferation (Fig. 1B). The CGFe obtained was a yellowish clear fluid, and each 50 ml blood sample produced 4 ml of CGFe. The cells were vimentin positive (Fig. 1C) and keratin negative (Fig. 1D) according to immunochemistry staining, indicating that these primary cells were of mesenchymal origin.
CGF enhances periodontal ligament cell proliferation. The proliferation of hPDLCs was measured with CCK-8 assays (Fig. 2). After 24, 48 and 72 h, CGFe significantly enhanced the proliferation of hPDLCs compared with the control group (P<0.01), while TNF-α markedly inhibited the proliferation of hPDLCs (for TNF-α vs. control, P<0.05 at 24 and 48 h; P<0.01 at 72 h). In addition, the proliferation rate of the CGFe group was significantly higher than that of the control group. Compared with the control group, the ALP activity in the TNF-α group was decreased. **P<0.01. CGFe, concentrated growth factor exudate; hPDLCs, human periodontal ligament cells; TNF-α, tumor necrosis factor-α; ALP, alkaline phosphatase; OD, optical density.

CGFe increases hPDLC proliferation. The proliferation of hPDLCs was measured with CCK-8 assays (Fig. 2). After 24, 48 and 72 h, CGFe significantly enhanced the proliferation of hPDLCs compared with the control group (P<0.01), while TNF-α markedly inhibited the proliferation of hPDLCs (for TNF-α vs. control, P<0.05 at 24 and 48 h; P<0.01 at 72 h).

CGFe increases ALP activity. After 7 and 14 days of culture, the hPDLCs cultured in the CGFe group showed the highest levels of ALP activity compared with the other experimental groups (Fig. 3). Treatment with TNF-α significantly inhibited the ALP activity of hPDLCs, as shown by comparison of the TNF-α group with the CGFe and CGFe+TNF-α groups, and the difference between these groups was significant (P<0.01) at both time-points. As expected, the ALP activity in all groups progressively increased over time.

Alizarin Red S staining and semi-quantification of mineralized matrix nodules. In order to detect the formation of mineralized matrix nodules, Alizarin Red S staining was performed (Fig. 4A-D). After 21 days of osteogenic induction, mineralized nodules were observed with an inverted phase-contrast microscope, and the number of nodules was notably higher in the CGFe group (Fig. 4C), compared with the control (Fig. 4A) or CGFe+TNF-α (Fig. 4D) groups. The TNF-α group (Fig. 4B) formed the fewest mineralized nodules.

Table I. Primers used for quantitative polymerase chain reaction analysis.

| Gene        | Forward (5’-3’) | Reverse (5’-3’)        | PCR products (bp) |
|-------------|-----------------|------------------------|-------------------|
| β-actin     | AGAAAATCTGGCACCACACC | GGGGTGGTGAAGGTCTAAA    | 139               |
| Osteocalcin | GCGGCTACCTGTATCAGGG | TCAGCCAACTCGTCACAGTC   | 106               |
| RUNX2       | CACCATGTCAGCACAACTTCTT | TCACGTGCTCATTGTGC      | 96                |
| Osterix     | TGCTTGAGGAGGAAGTTCAC | AGGTCACTGCCCACAGAGTA   | 148               |

RUNX2, runt-related transcription factor 2; bp, base pairs.

**P<0.01. CGFe, concentrated growth factor exudate; hPDLCs, human periodontal ligament cells; TNF-α, tumor necrosis factor-α; ALP, alkaline phosphatase; OD, optical density.
The mineralized matrix nodules were quantified after 21 days of induction (Fig. 4E). The absorbance values at 570 nm revealed that extracellular calcium deposition in the CGFe group was significantly higher than the control or CGFe+TNF-α groups (P<0.01).

**CGFe increases osteogenic-associated gene and protein expression.** The gene expression of the RUNX2, OSX and OCN was determined after 4, 7 and 14 days of osteogenic induction (Fig. 5). It was observed that on days 4, 7 and 14, the expression of RUNX2 and OSX in the TNF-α (10 ng/ml) groups was decreased compared with the control group (P<0.01; excluding OSX expression on day 7, P<0.05), and the expression of these two genes in the CGFe+TNF-α group showed no significant increase compared with the control group. By contrast, on days 4, 7, and 14, expression of the RUNX2 and OSX genes in the CGFe group was significantly increased, compared with the control group (P<0.01; Fig. 5A and B). The expression of the downstream OCN gene was not significantly different between groups on day 4. By day 7, the CGFe group began to surpass the control group (P<0.01). After 14 days of culture, OCN expression in the TNF-α group was lower than that of the control group (P<0.01; Fig. 5C).

Western blotting was performed to examine the effects of CGFe on hPDLC differentiation. hPDLCs were cultured in each medium for 14 days. As shown in Fig. 6A, compared
with the control group, the protein expression of RUNX2 was upregulated in the CGFe group, and downregulated in the CGFe+TNF-α and TNF-α groups (P<0.01; Fig. 6B). A similar trend was observed for OSX expression. Although the protein level of OSX was upregulated in the CGFe+TNF-α group, there was no significant difference compared with the control group (P>0.05; Fig. 6B).

**Discussion**

CGFe can be obtained from the venous blood of patients and thus is convenient and economical to prepare. CGFe contains high concentrations of growth factors and provokes no immune reaction (8-10,22,25). According to previous studies (4-8), the CGFe obtained from different patients in the present study contained different inventories of components, yet the major components were the same. These included epidermal growth factor, platelet-derived growth factor, transforming growth factor (TGF-β), vascular endothelium growth factor, insulin-like growth factor, bone morphogenetic protein (BMP), and fibroblast growth factor (4-8). In the last decade, CGFe has been widely used in the reconstruction of bone tissue in the surgical field (8,9). These growth factors have functions in accelerating the revascularization of injured tissues and inducing the migration, proliferation, and differentiation of fibroblasts and osteoblasts (8-10,25).

TNF-α is a common and crucial inflammatory factor in the development of periodontal inflammation and alveolar bone resorption (20). It has been established that TNF-α significantly inhibits the proliferation of hPDLCs and mesenchymal stem cells (5,26,27). In addition, activation of the nuclear factor (NF)-κB signaling pathway inhibits the expression of RUNX2, which is a downstream transcription factor induced...
by the exogenous BMP2-mothers against decapentaplegic homolog (Smad) signaling pathway controlling osteoblast differentiation, thus inhibiting the formation of bone (28).

In the present study, TNF-α (10 ng/ml) was used to construct an inflammatory microenvironment. It was demonstrated TNF-α reduced ALP activity, mineralization ability and the expression of RUNX2 and OSX, compared with the control group. This result was concordant with the findings of Yang et al. (18), which revealed that the bone formation efficiency of gingival mesenchymal stem cells and periodontal ligament stem cells decreased under TNF-α-induced inflammatory conditions.

Previous studies have shown that RUNX2 and OSX are two essential transcription factors in the osteogenic pathway (29,30). In particular, RUNX2 regulates cell osteogenic differentiation and serves a central role in multiple osteogenic signaling pathways (29). OSX is an osteogenic-specific transcription factor with a zinc finger structure. A previous study revealed that no bone formation occurs in mice lacking the OSX gene (30). The level of ALP activity reflects the tendency of cells to transform in the osteogenic direction midway during the osteoprogenitive process, while OCN is a late marker of osteogenesis. OCN is the most abundant non-procollagen protein in bone tissue and promotes osteogenic differentiation by combining with minerals (31,32). Positive Alizarin Red S staining of the mineralized nodules formed indicates the expression of OCN and osteogenesis at a later stage (31,32).

In the present study, CGFe was added to cell cultures to observe the osteogenic efficiency of hPDLCs, by detecting the expression of RUNX2, OSX and OCN genes, as well as ALP activity and mineralized nodule formation in each group of hPDLCs. The formation of mineralized nodules was used to determine the osteogenic capacity of each group of hPDLCs. It was observed that even in the presence of inflammatory cytokine stimulation, hPDLCs cultured with CGFe still exhibited osteogenic differentiation and mineralization ability. By comparing the assay and imaging results of the CGFe group with the CGFe+TNF-α group, it was demonstrated that ALP activity in the CGFe group was stronger than that of the CGFe+TNF-α group, and the formation of mineralized nodules was notable in both groups, although the CGFe group was stained more prominently. In addition, ALP activity in the CGFe+TNF-α group was significantly higher than the TNF-α group, and the CGFe+TNF-α group had deep mineralized staining with a large area and high density, while no mineralized nodule formation was observed in the TNF-α group.

The western blotting results of the present study revealed that RUNX2 and OSX expression was higher in the CGFe group, compared with the CGFe+TNF-α group, indicating that TNF-α may play an inhibitory role upstream of protein translation. By contrast, growth factors contained in the CGFe may antagonize the inhibitory effect of TNF-α on cell proliferation. For example, TGF-β1 induces the phosphorylation of Smad2/Smad3, which upregulates RUNX2 transcription (29), thereby impeding TNF-α activation of the NF-κB signaling pathway, which would result in RUNX2 inhibition.

In conclusion, CGFe not only has an osteogenic effect on hPDLCs in a normal culture, but also promotes hPDLC osteogenesis in a TNF-α-induced inflammatory microenvironment. In the present work, a single inflammatory factor, TNF-α, was used to mimic the microenvironment of periodontal disease, and this imposed certain limitations. The enhancing effect of CGFe on osteogenic differentiation of hPDLCs stimulated by other inflammatory factors will be examined in follow-up experiments. To study the effects of CGFe in the treatment of alveolar bone defects, in vivo should be performed. In addition, the exact mechanism of how CGFe enhanced the proliferation and osteogenic differentiation of hPDLCs should be determined.

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Availability of data and materials

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

Authors' contributions

XL, HY and BW conceived and designed the study. XL, YZ, and HL performed cell culture, immunostaining and proliferation analysis. XL and YZ performed the experimental procedures of osteogenic differentiation induction, RT-qPCR, and western blotting. HY, ZZ and ZY provided reagents and interpreted the data. XL, HY and BW performed data analysis and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Jilin University Health Science Center (Changchun, China). Written consent was obtained prior to experimentation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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