

Effect of concentrated growth factors on beagle periodontal ligament stem cells *in vitro*

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Abstract. Identifying a reliable and effective cytokine or growth factor group has been the focus of stem cell osteogenic induction studies. Concentrated growth factors (CGFs) as the novel generation of platelet concentrate products, appear to exhibit a superior clinical and biotechnological application potential, however, there are few studies that have demonstrated this effect. This study investigated the proliferation and differentiation of periodontal ligament stem cells (PDLSCs) co-cultured with CGFs. The rate of proliferation was analyzed by cell counting and an MTT assay. Mineralization nodule counts, alkaline phosphatase activity detection, qPCR, western blot analysis and immunohistochemistry were used to analyze mineralization effects. The results showed that CGF significantly promoted the proliferation of PDLSCs, and exhibited a dose-dependent effect on the activation and differentiation of the stem cells. The application of CGF on PDLSC proliferation and osteoinduction may offer numerous clinical and biotechnological application strategies.

Introduction

The increased lifespan of human beings has led to the development of novel therapeutic strategies aiming to preserve aged tissue, which range from the replacement of lost or injured tissues to regeneration of the damage tissue (1). Regenerating alveolar bone as an alternative therapy for periodontal regeneration has been increasingly used in dentistry. A deficient alveolar ridge not only fails to provide sufficient support and retention for teeth or dentures, but also hinders dental implant placement (2). The finding of stem cells was a medical breakthrough and led to the novel field of medicine entitled regenerative medicine (3). Postnatal stem cells have

been isolated from various tissues, including dental tissue (4). Seo *et al* suggested that the human periodontal ligament (PDL) contains a population of postnatal multipotent stem cells (5). Stem cells require specific microenvironments for survival (6), thus, in theory periodontal ligament stem cells (PDLSCs) are the most direct and reliable source for periodontal tissue regeneration. Although PDLSCs are easy to separate and purify, the number of cells required for bone regeneration is unachievable in a short period of time. After several passages, it is difficult to induce the differentiation of cells that are prone to aging and changes in phenotype (7,8). Thus, an effective way to rapidly amplify PDLSCs is a current focus of numerous studies.

Growth factors stimulate the differentiation of mesenchymal cells into osteoblasts and accelerate the osteogenesis of these cells. The growth factors that are known to be associated with bone formation are bone morphogenetic protein (BMP), transforming growth factor (TGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and epidermal growth factor (EGF) (9-11). When these growth factors are used alone, there are often certain shortcomings. For example, when using BMP-2 alone, it has a short half-life and is susceptible to proteolytic degradation. When the concentration is too high, it may lead to tissue edema, an increased inflammatory response, and prevention of novel bone formation (12-14). In addition, the human body is a complex biological environment; thus, stem cell osteogenic differentiation is not regulated by one growth factor alone. Therefore, it is essential to identify an autologous source and effective cytokine or growth factor group in stem cell osteogenic induction studies. Platelet-rich plasma (PRP) (15), a platelet concentrate product, contains numerous growth factors. Previously, it was demonstrated that the PRP may induce proliferation, but inhibits the differentiation of PDLSCs (16). The application of PRP has also been controversial as the addition of dissimilar thrombin and anticoagulant during preparation may pose risks of immune rejection and transmission of infectious diseases. The platelet-rich fibrin (PRF), the second generation of platelet concentrate products (17), exhibits the same properties as PRP with the advantages of an osteogenic ability, a simple preparation process, and lack of added biological agents, as it is produced from autologous blood. Concentrated growth factors (CGF) were developed by Sacco in 2006. It is produced by a centrifuge device (Medifuge Silfradent srl, Italy), similar to the production of PRF (18). The different centrifugation speed

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permits the isolation of fibrin matrix that is markedly larger, denser and richer in growth factors as compared to PRF. In theory, CGFs appear to exhibit superior clinical and biotechnological application potential (19); however, there are few studies supporting this.

The aim of this study was to analyze the *in vitro* biological effects of CGFs on the proliferation and differentiation of canine PDLSCs, and to investigate whether this effect occurs in a dose-dependent manner.

Materials and methods

Isolation of beagle PDLSCs and preliminary identification.

One healthy beagle dog (18 months old, 14.5 kg, male) was supplied by the Laboratory Center of the Second Military Medical University of the Chinese PLA (No. SCXK-Shanghai 2012-0003). The entire experimental procedure was in accordance with the Regulations of the Administration of Affairs Concerning Experimental Animals formulated by the Ministry of Science and Technology of China. Beagle PDLSCs were isolated and cultured as described previously (4,20,21). Briefly, periodontal ligament tissues were scraped from the intermediate 1/3 of the root. Collagenase type I (3 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) and dispase II (4 mg/ml; Roche, Basel, Switzerland) were added at a 1:1 mixture and digested for 1 h at 37°C. The mixture was passed through a 70 µm cell sieve (BD Biosciences Franklin Lakes, NJ, USA), using α-minimal essential medium (MEM) containing 15% fetal bovine serum (FBS) to adjust the cell density to 1x10⁴/ml. The cells were then incubated in a 5% CO₂ atmosphere at 37°C. Following expansion, the STRO-1⁺ cells (Biolegend, San Diego, CA, USA) were separated by flow cytometry. The cells were then inoculated on α-MEM growth medium (Gibco-BRL, Carlsbad, CA, USA) containing 20% FBS (Gibco), 2 mM L-glutamine, 100 µM L-ascorbate-2-phosphate, 1 mM sodium pyruvate, 50 U/ml penicillin and 50 µg/ml streptomycin (21). The logarithmic growth phase cells underwent osteogenic, chondrogenic and adipogenic multi-induction experiments, and the induced cells were stained on day 21 with Alizarin Red S (Sigma-Aldrich), Alcian blue (Sigma-Aldrich) and Oil Red O (Sigma-Aldrich) accordingly.

CGF preparation. It was essential to use cultured PDLSCs and CGFs from the same donor. Under sterilized conditions, venous blood was drawn from the beagle forearm, divided into sterile Vacuette tubes (1 and 3 ml samples in each tube, respectively) without anticoagulants and immediately placed in a centrifuge for centrifugation. The built-in program was: 30" acceleration, 2' 408 x g, 4' 323 x g, 4' 408 x g, 3' 3,000 rpm, and 36" deceleration and stop. The fresh whole blood was divided into three layers (Fig. 1A). The intermediate filament protein gel layer was collected and pressed onto membranous film (MF200, Wisdom, Beijing, China; Fig. 1B).

Experimental groups. There were 6 groups used in the present study, the standard group (0 CGF), standard group + 1 CGFs (prepared with 1 ml fresh whole blood), standard group + 3 CGFs (prepared with 3 ml whole blood), osteogenesis induced fluid (0 CGFs), osteogenesis induced fluid + 1 CGF and osteogenesis induced fluid + 3 CGFs. CGFs were added on the first

Table I. List of primers.

Gene	Sequences (5'-3')
BSP	F: CGATTTCCAGTTCAGAGCAGTAGT R: CAGCGTCCGATTCATCTTCAT
Collagen I	F: TGGGGCAAGACAGTGATCG R: GGAGGGAGTTTACAGGAAGCAG
OCN	F: GCTGTGGCCGCACTCTGC R: AGAGTGGGGCTGGCCGCTC
GAPDH	F: AAGGTCGGAGTCAACGGATTT R: GGTTCACGCCCATCACAAA

BSP, bone sialoprotein 2; OCN, osteocalcin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

day and experimental check points were at days 3 (D3), 7, 14 and 21 following inoculation.

The standard culture medium was composed of α-MEM growth medium as described previously. The osteogenic medium (21) was composed of α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 µM L-ascorbate-2-phosphate, 10⁻⁷ M dexamethasone, 1.8 mM inorganic phosphate (KH₂PO₄), 50 U/ml penicillin and 50 µg/ml streptomycin.

Cell growth and proliferation analysis. PDLSC proliferation increased following CGF treatment and was analyzed by cell counting and an MTT assay. The absorbance value of each well was measured at 490 nm by the microtiter analyzer (Multiskan FC; Thermo, Vantaa Finland). According to the measured OD values, with time as the x-axis and absorbance value as the y-axis, the cell growth curve was plotted.

Osteoinduction assessment. The mineralized nodules were counted by Alizarin Red S (Sigma-Aldrich, St. Louis, MO, USA) staining and mineralized nodule formation was observed under the microscope (BX43; Olympus, Tokyo, Japan).

Alkaline phosphatase (ALP) activity detection was conducted using a commercialized reagent cartridge (? , USA) at each experimental check point and was analyzed in the microtiter analyzer at OD 405 nm. The ALP activity was expressed as nU/cell and mU/plate.

qPCR was conducted using TRIzol reagent to extract RNA, and using a reverse transcription kit (Takara, Tokyo, Japan) to synthesize cDNA. Primers are listed in Table I. A two-step amplification response procedure was selected with specifications of 95°C for 120 sec, 95°C for 15 sec and 61°C for 40 sec for 40 cycles.

For the western blot analysis, RIPA lysis buffer was added, homogenated and centrifuged and the protein supernatant was collected for sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes, sealed and incubated with primary [BSP (Abcam, Cambridge, MA, USA), Col-I (Abcam) and OCN (Abcam)] and secondary antibodies (ZSGB-Bio, Beijing, China). The membranes were washed and immunoblot chemiluminescence detection reagents (enhanced chemiluminescence, ECL), were

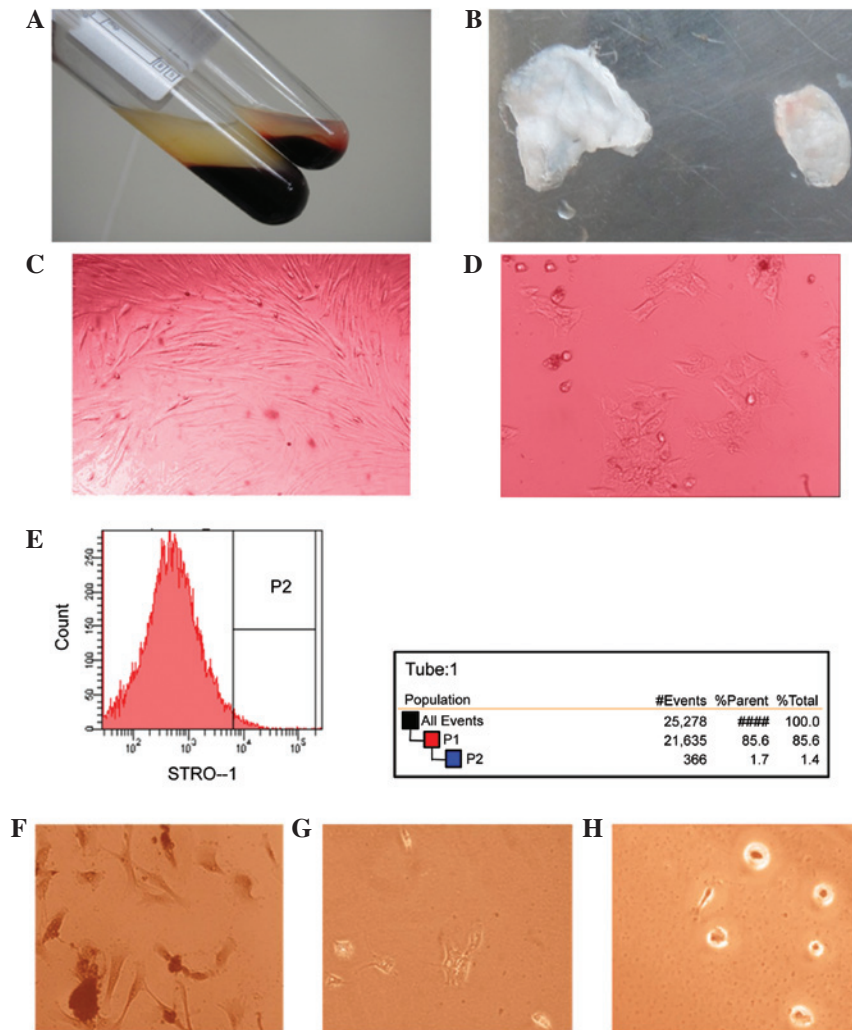


Figure 1. CGFs were prepared with (A) 1 and 3 ml fresh whole blood and CGFs were pressed into (B) membranous film. (C) Primary PDLSCs were obtained from beagle periodontal ligament; and (D) STRO-1⁺ PDLSCs were isolated by a flow cytometry. (E) The separation rate by flow cytometry was 1.7%. PDLSCs were divided into osteogenic, chondrogenic and adipogenic induction groups and stained by (F) Alizarin Red S, (G) Alcian blue and (H) Oil Red O. The result showed a positive reaction. CGFs, concentrated growth factors; PDLSCs, periodontal ligament stem cells.

used for exposure. Glyceraldehyde 3-phosphate dehydrogenase protein served as a system internal reference and stripe gray values were measured. The results were compared based on the differences in the expression of purpose/internal reference ratio.

For immunohistochemical detection, cell climbing film was produced, sealed and incubated with primary and secondary antibodies. DAB, chromogenic and hematoxylin staining was conducted, and the samples were sealed and observed under the microscope.

Statistical analysis. Cell count, ALP activity, mineralized nodule numbers and the positive expression rate of immunohistochemical staining were presented as the mean \pm SE and compared with the control group.

For the MTT assay, the survival capacity of the control group was set as 100%. The experimental groups were calculated as percentages of the control group. $P < 0.05$ was considered to indicate a statistically significant difference.

Relative quantification of qPCR results were obtained using the comparative Ct method. According to the equation

$\text{Fold} = 2^{-\Delta\Delta C_t}$, the differences in the relative expression of the target gene of the experimental and control groups were calculated.

Western blot analysis results of three experiments were compared with the protein expression differences by scanning the striped gray value.

Results

Cell culture, activity and proliferation. Flow cytometry was used to screen PDLSCs (Fig. 1D) from PDLSCs (Fig. 1C); the separation rate was 1.7% (Fig. 1E). To assess the differentiation capacity of PDLSCs, the separated PDLSCs were divided into osteogenic, chondrogenic and adipogenic induction groups. The cells were then stained by Alizarin Red S (Fig. 1F), Alcian blue (Fig. 1G) and Oil Red O (Fig. 1H). The results showed a positive reaction which indicated that the PDLSCs exhibited stem cell-specific differentiation ability.

Fig. 2A shows the proliferation effects of CGFs on PDLSCs. CGFs were identified to increase PDLSC proliferation in a

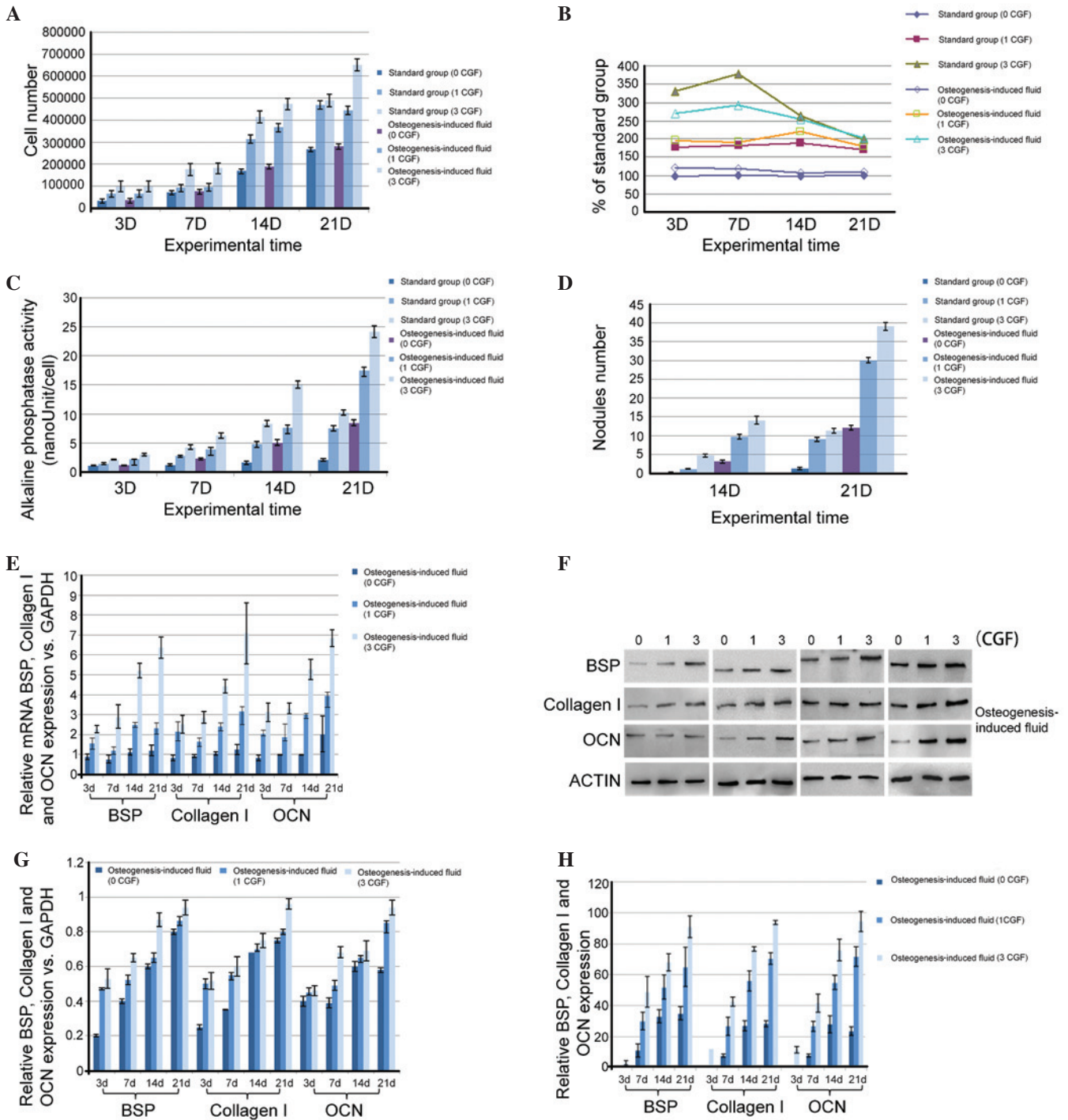


Figure 2. (A) Proliferation assessment of beagle PDLSCs in six different experimental conditions and at four experimental times. (B) MTT assay results of the test groups are expressed as mean percentages of the control. (C) Alkaline phosphatase activity quantification (nU/cell), and (D) the numeration of critical sized mineralization nodules in beagle PDLSCs cultures in six different experimental conditions. Relative expression of BSP, collagen I and OCN with osteogenesis-induced fluid based on (E) qPCR, (F and H) western blot analysis and (G) immunohistochemistry. Results are expressed as the mean \pm SD. PDLSCs, periodontal ligament stem cells; BSP, bone sialoprotein 2; OCN, osteocalcin.

time- and dose-dependent manner ($P < 0.05$), and a significant increase appeared at 14 day. From the MTT assay, in standard culture conditions, the stimulation of proliferation ranged from 178 to 189% with 1 CGF and from 198 to 330% with 3 CGF (Fig. 2B). The stimulation level was stable with 1 CGF, but the cell activity peaked on day 7 with 3 CGF. The cultures with 3 CGF were more strongly stimulated compared with those

with 1 CGF ($P < 0.05$) at least up to 14 days. In the osteogenic medium, the stimulation of proliferation ranged between 178 and 196% with 1 CGF and between 201 and 292% with 3 CGF (Fig. 2B). The stimulation level was stable with 1 and 3 CGF. In addition, it was observed that the cultures with 3 CGF were more strongly stimulated than those with 1 CGF ($P < 0.05$) throughout the experimental period.

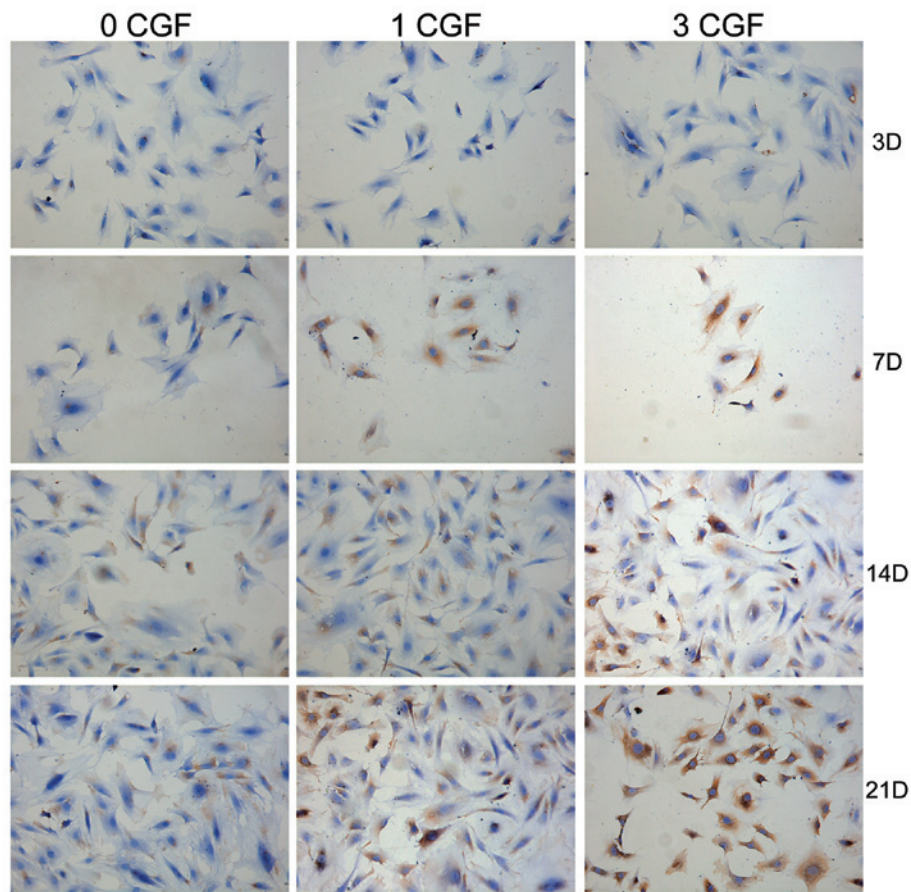


Figure 3. Relative expression of bone sialoprotein 2 BSP with osteogenesis-induced fluid observed by immunohistochemical analysis. DAB, chromogenic and hematoxylin staining was conducted. Bar, 100 μ m. CGFs, concentrated growth factors.

Osteoinduction. ALP activity detection and mineralized nodule counts were performed to determine the effect of CGFs on the induction of osteogenic differentiation of PDLSCs cells. ALP activity was detected on days 3, 7, 14 and 21, and mineralized nodule counts were conducted on days 14 and 21. In the presence of 1 or 3 CGFs, the ALP activities (Fig. 2C) and the number of mineralized nodules (Fig. 2D) were significantly higher ($P < 0.05$) than the values of the respective control groups, and stimulation of differentiation was significantly higher ($P < 0.05$) with 3 CGF than with 1 CGF, regardless of the culture conditions.

The experiments performed showed that the CGFs exhibits a significant positive effect on PDLSC cell morphology due to osteogenic induction and differentiation. To the best of our knowledge, no previous study has investigated this event at the molecular level. In the present study, qPCR analysis was used to detect the expression of bone sialoprotein 2 (BSP), collagen I and osteocalcin (OCN) of different groups at each experimental check point during osteogenic induction. In Fig. 2E, the mRNA expression level of BSP showed an increasing trend along with the increasing quantity of CGF added ($P < 0.05$). In addition, as the culture time increased, BSP mRNA expression was increased, however, it showed no significant increase for the first 7 days ($P > 0.05$) and then significantly increased on day 14 ($P < 0.05$). OCN and collagen I mRNA expression also showed the same increasing trend following CGF addition.

Furthermore, western blot analysis was used to analyze cell growth on the protein level. As shown in Fig. 2F, CGFs were observed to upregulate BSP protein expression in PDLSCs during the culture period ($P < 0.05$). The quantitative measurement is shown in Fig. 2G. BSP protein expression in cells cultured in osteogenic medium was significantly increased with 3 CGF ($P < 0.05$) on day 7 and 21 compared with 1 CGF. Collagen I protein expression levels also increased in a time- and dose-dependent manner, and peaked on day 14 ($P < 0.05$). The OCN protein expression levels increased with time and peaked at day 21 ($P < 0.05$).

Furthermore, immunohistochemical analysis of the flag factor BSP, collagen I and OCN protein expression was also performed during the osteogenic induction process at different experimental check points in all the experimental groups. As shown in Figs. 3-5, BSP, collagen I and OCN-positive expression, respectively, occurred at day 7 when co-cultured with CGF; however, the control group showed a positive expression at day 14. The results indicated that BSP, collagen I and OCN-positive expression increased (Fig. 2H) in a time- and dose-dependent manner ($P < 0.05$).

Discussion

Adult stem cells are specific to adult tissues and organs. Under normal circumstances, the majority of the cells are in a resting state; however, damage to body tissues stimulates

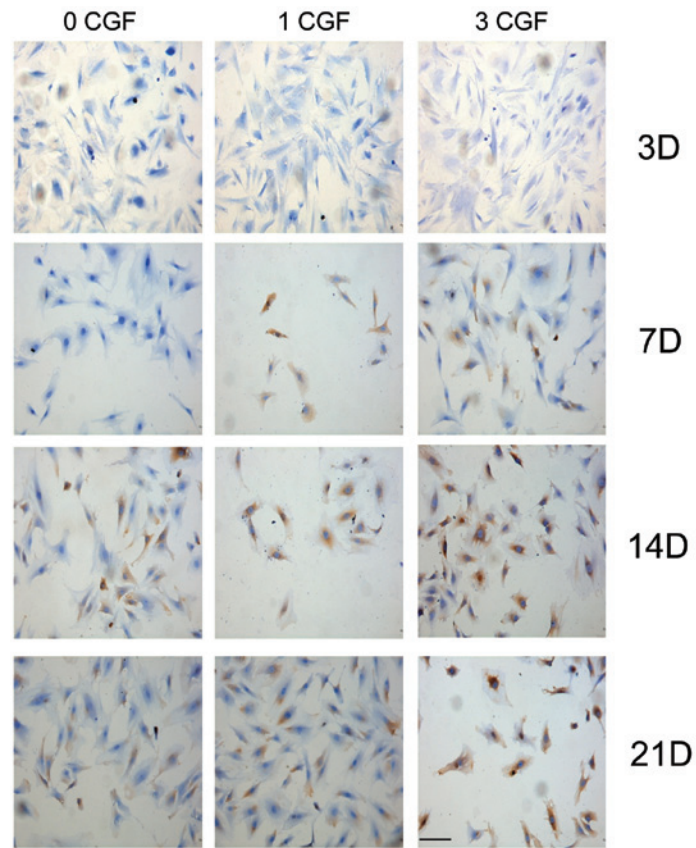


Figure 4. Relative expression of collagen I with osteogenesis-induced fluid observed by immunohistochemical analysis. DAB, chromogenic and hematoxylin staining was conducted. Bar, 100 μ m. CGFs, concentrated growth factors.

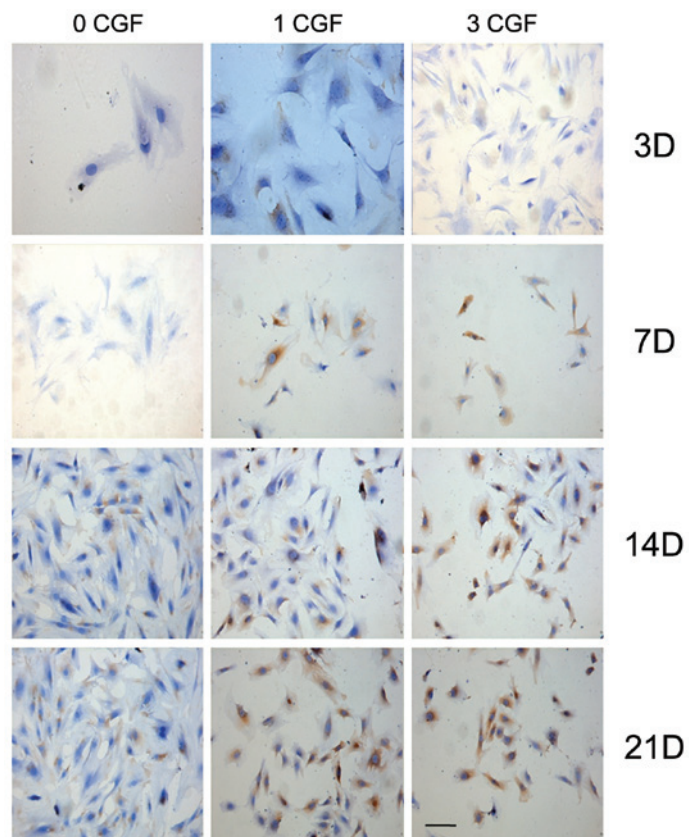


Figure 5. Relative expression of osteocalcin with osteogenesis-induced fluid observed by immunohistochemical analysis. DAB, chromogenic and hematoxylin staining was conducted. Bar, 100 μ m. CGFs, concentrated growth factors.

differentiation of the stem cells in the tissue. The stem cells differentiate into the damaged tissue cells and are involved in tissue repair and reconstruction. Therefore, in theory PDLSCs are the most direct and reliable seed cells for periodontal tissue regeneration. There is no ideal method for the isolation of adult stem cells. Generally, stem cells are separated and identified based on their colony-forming ability and specific biological and physical characteristics. As no specific markers have been identified for PDLSCs, they are predominantly identified by STRO-1, CD146 and other mesenchymal stem cell marker proteins and are isolated by the immunomagnetic bead separation method or flow cytometry. These methods are the same for bone marrow stem cells and dental pulp stem cells, which have similar organizational structure and embryonic origin to PDLSCs (21). As an increasing number of PDLSC studies have been conducted, STRO-1, CD146 and other mesenchymal stem cell surface markers and certain perivascular cell surface markers, such as CD105, CD106 and CD166, have been widely recognized. The STRO-1 antibody immunomagnetic or the flow cytometry separation methods are commonly known to be the most effective separation methods for PDLSCs (4,20-23). In the present study, STRO-1⁺ cells were separated by flow cytometry and preliminary identification indicated that these cells possess high self-renewal capacity and multi-lineage differentiation potential.

Similar to PRF, the CGFs were only centrifuged once to avoid the loss of fibrinogen. The CGFs formed free modification gelatinous fiber blocks, which were convenient for operation and application. In the present study, CGFs were able to significantly stimulate PDLSC proliferation in a dose-dependent manner *in vitro*, in standard or differentiation medium. The mechanism responsible for the cell proliferation by CGFs may be explained as follows: CGFs are rich in a variety of growth factors, such as TGF- β , PDGF-AB, VEGF and IGF-I (24). These growth factors while functioning on their own (25-29), are also synergistic and create close contact tissue repair regulatory systems (24,30,31). Unlike PRP, CGFs do not dissolve rapidly following application, instead, the strong fibrin gel in the matrix addition is slowly remodeled in a similar manner to a natural blood clot. Thus, CGFs prolonged the duration of growth factor action, which is conducive for the growth factor synergy (32-34), and enhances cell proliferation and osteogenic differentiation. Previous studies have demonstrated that the PRP may induce proliferation, but inhibits differentiation (16), and certain studies have indicated that high concentrations of PRP may also exhibit an inhibitory effect on the cultured cells (35). In the present study, this was not observed. To avoid any bias related to immune incompatibility, in the present study, CGF was obtained from the same donor as for the PDLSCs. CGFs exhibited no cytotoxic effect on the PDLSCs at the two doses tested similar to PRF (36). CGF also significantly promoted the proliferation of PDLSCs, and exhibited a dose-dependent effect on the activation and differentiation of the stem cells.

With the aid of the osteogenic inductive medium, PDLSCs develop osteogenically. The cells undergo multiple independent stages of differentiation in order to evolve into osteoblasts. The process includes: The conversion phase, the proliferation phase, cell aggregation secretory phase and extracellular matrix calcification period (37). Throughout the differentiation

process, the alkaline phosphatase activity, matrix mineralization, osteocalcin and collagen I are all specific markers for stem cell osteogenic differentiation. Osteoblasts synthesize different products in different stages. Collagen I is produced in the cell proliferation phase, cell aggregation secretory phase synthesizes alkaline phosphatase, and osteocalcin is produced in the extracellular matrix calcification phase (38). Thus, in the present study, the indicators that determine osteoblast differentiation of PDLSCs were mineralized nodule formation, alkaline phosphatase activity, type I collagen synthesis and specific protein expression, such as BSP and OCN. In the present study, ALP activity and mineralized nodule count were elevated by CGF in a time- and dose-dependent manner. qPCR, western blot analysis and immunohistochemical results were used to determine that CGF mediated the osteogenic differentiation of PDLSCs and the results show that CGF accelerates osteogenesis during the differentiation process. Similar the results have been demonstrated previously (39); thus, CGF may contribute to the differentiation of PDLSCs.

In conclusion, CGF significantly promotes the proliferation of PDLSCs and has a dose-dependent effect. In standard culture medium, CGF induces PDLSC osteogenic differentiation. In conditioned medium, CGFs significantly accelerate the osteogenesis transformation process of PDLSCs. CGF mediated culture medium either directly induces transformation or accelerates transformation in a dose-dependent manner. Future studies are required to identify the optimal induction dose of CGF, to determine the mechanism underlying the dose-dependent trend and to reproduce the results in *in vivo* studies. The present study has therefore provided an experimental basis for further PDLSCs clinical applications and studies.

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