Effects of concentrated growth factor on proliferation, migration, and differentiation of human dental pulp stem cells in vitro

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Abstract
Concentrated growth factor, a novel autologous plasma extract, contained various growth factors which promoted tissue regeneration. In this study, we aimed to investigate the biological effects of concentrated growth factor on human dental pulp stem cells. The microstructure and biocompatibility of concentrated growth factor scaffolds were evaluated by scanning electron microscopy. Cell proliferation and migration, odontoblastic and endothelial cell differentiation potential were assessed after exposing dental pulp stem cells to different concentrations (5%, 10%, 20%, 50%, or 80%) of concentrated growth factor extracts. The results revealed that concentrated growth factor scaffolds possessed porous fibrin network with platelets and leukocytes, and showed great biocompatibility with dental pulp stem cells. Higher cell proliferation rates were detected in the concentrated growth factor–treated groups in a dose-dependent manner. Interestingly, in comparison to the controls, the low doses (<50%) of concentrated growth factor increased cell migration, alkaline phosphatase activity, and mineralized tissue deposition, while the cells treated in high doses (50% or 80%) showed no significant difference. After stimulating cell differentiation, the expression levels of dentin matrix protein-1, dentin sialophosphoprotein, vascular endothelial growth factor receptor-2 and cluster of differentiation 31 were significantly upregulated in concentrated growth factor–supplemented groups than those of the controls. Furthermore, the dental pulp stem cell–derived endothelial cells co-induced by 5% concentrated growth factor and vascular endothelial growth factor formed the most amount of mature tube-like structures on Matrigel among all groups, but the high-dosage concentrated growth factor exhibited no or inhibitory effect on cell differentiation. In general, our findings confirmed that concentrated growth factor promoted cell proliferation, migration, and the dental pulp stem cell–mediated dentinogenesis and angiogenesis process, by which it might act as a growth factor–loaded scaffold to facilitate dentin–pulp complex healing.

Keywords
Concentrated growth factor, dental pulp stem cells, odontoblastic, endothelial, pulp regeneration

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Introduction
Due to incomplete root development, young permanent teeth with devitalized dental pulp face a high risk of tooth fracture and subsequent tooth loss.1 In recent years, apical revascularization has been an alternative dental treatment for necrotic immature teeth. Apical closure and increased root length can be observed clinically after the revascularization approach, while the efficiency of preserving tooth biological functions is limited.2 Currently, tissue engineering technology has been regarded as a promising strategy of regenerative endodontics.3

Growth factors are the driving force for tissue regeneration by regulating many aspects of cellular behavior, the function of which has been widely accepted.4 For example,
transforming growth factor-beta (TGF-β) and insulin-like growth factor promote cell proliferation; TGF-β and vascular endothelial growth factors (VEGF) enhance cell migration; bone morphogenetic proteins (BMPs) and fibroblast growth factor 2 (FGF2) stimulate osteogenic differentiation; VEGF and platelet-derived growth factor (PDGF) are essential in the process of angiogenesis. Native growth factors are embedded within the extracellular matrix (ECM). However, exogenous growth factors applied alone in tissue engineering have a short life due to rapid proteolysis.

Biomimetic scaffold is another key element of tissue engineering, which mimics the ECM of normal pulp. Platelet concentrates are attractive autologous scaffolds suitable for regenerative medicine as its fiber architecture and rich growth factors. Concentrated growth factor (CGF) is the third generation of autologous plasma extract prepared by a special centrifugal program. CGF scaffolds possess unique three-dimensional (3D) fibrin networks, which may establish a conducive microenvironment for newly formed tissue growing inwards. Particularly, the optimized manufacturing process endows CGF with a higher level of growth factors, platelets, and cytokines than the traditional platelet concentrates such as platelet-rich plasma (PRP) and platelet-rich fibrin (PRF).

In previous studies, CGF has been suggested as potentially ideal scaffolds for bone defect repair due to its osteogenic promotion effect on bone marrow stem cells (BMSCs). Moreover, recent studies investigated that CGF promoted the proliferation and migration activity of periodontal ligament stem cells (PDLSCs) and Schwann cells (SCs) in vitro, and CGF treatment led to functional nerve recovery in the sciatic nerve injury rat model.

To the best of our knowledge, currently there is an absence of research investigating the possible utility of CGF in dental pulp regeneration. Therefore, in this study, we aimed to test this hypothesis by assessing its cytocompatibility and detecting cell proliferation and migration activity and odontoblastic and endothelial differentiation capacity after treating dental pulp stem cells (DPSCs) under different concentrations of CGF extracts.

**Materials and methods**

**Cell isolation and culture**

All research protocols in this study were approved by the Ethics Committee of School of Stomatology, Wuhan University, China. Human DPSCs were isolated and identified as described in our previous studies. Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; HyClone, Logan, UT, USA) supplemented with 100 U/mL penicillin/streptomycin (P/S; HyClone) and 10% (v/v) fetal bovine serum (FBS; Biological Industries, Kibbutz Beit-Haemek, Israel) at 37°C with an atmosphere of 5% CO₂ and 95% air. The cell culture medium was changed every other day. DPSCs between passages 3 and 5 were used in the following experiments.

**Preparation of CGF scaffolds and extracts**

Venous blood was collected from eight healthy volunteers using sterile VACUETTE tubes without additive (Greiner Bio-One, Kremsmünster). Then the tubes with whole blood (9 mL sample in each tube) were immediately centrifuged by Medifuge MF200 (Silfradent, Santa Sofia) at fixed temperature. After centrifugation, CGF gel represented as the buffy coat in the middle layer and was carefully isolated from the red blood cell clots. The fluids inside the CGF gel were gently squeezed out by a special stainless steel compressor, and the gel was pressed into CGF membranes as scaffolds.

For in vitro cell treatment, CGF extracts were produced by soaking each CGF gel into 9 mL blank DMEM at 37°C for 2 weeks. The obtained medium was defined as 100% CGF. Then 100% CGF was diluted with DMEM into 80%, 50%, 20%, 10%, or 5% (v/v) CGF as required.

**CGF scaffold characterization**

CGF scaffolds were cut into small pieces approximately 1 mm × 1 mm × 1 mm. A total of 10,000 DPSCs were seeded on each disk and incubated at 37°C for 7 days. Samples were fixed with 2.5% glutaraldehyde solution (Sigma-Aldrich, St Louis, MO, USA) at 4°C for 1 h and then dehydrated by gradient ethanol. Ultrastructure properties of CGF scaffolds and its biocompatibility with DPSCs were assessed by scanning electron microscopy (SEM; Sirion-FEG, Phillips, Eindhoven, The Netherlands). Histomorphology of CGF fibrin scaffolds was examined by H&E staining according to the standard protocol.

**Cell proliferation test**

**Cell Counting Kit-8 assay.** DPSCs at passage 4 were prepared into 96-well plates (1000 cells/well). After cell adherence, the medium was refreshed and different doses (5%, 10%, 20%, 50%, or 80%) of CGF extracts or 10% FBS were added, respectively. Cells in the control group were treated in blank DMEM. At different time points (1, 3, 5, and 7 days of culture), the optical density (OD) at 450 nm was measured according to the instruction of Cell Counting Kit-8 (CCK8; Dojindo, Kumamoto, Japan). Five independent biological replicates were assessed. Cell proliferation rate in each group was obtained after normalization with its mean OD value of day 1.
5-ethyl-2'-deoxyuridine incorporation assay. DPSCs were seeded over cover slips and treated, respectively, under different concentrations of CGF extracts for 3 days. Following the manufacturer’s guidelines, the cells were incubated in 50 μM 5-ethyl-2'-deoxyuridine (EdU) agent (RiboBio Co., Ltd, Guangzhou, China) for 3 h at 37°C to mark the proliferative cells, with 4',6-diamidino-2-phenylindole (DAPI) indicating total cell nuclei. Images were photographed by a fluorescent microscope (Leica, Wetzlar). The ratio of EdU-positive cells/DAPI-positive cells was calculated for statistical analysis.

Analysis of cell migration
After the cells were treated with serum starvation overnight, scratches were made by a 200-µL pipette tip. Medium was then changed into different concentrations (10%, 20%, 50%, or 80%) of CGF-conditioned medium, with blank DMEM as the control. Images were captured at 0 and 24 h after scratch making by a phase contrast microscope (Olympus, Tokyo, Japan). Relative cell migration rate was calculated using the following equation: \((\frac{(C_0 - E_24)}{(E_0 - C_24)})\) \times 100% with \(C_0\) and \(C_{24}\) respectively, representing the scratch areas at the beginning and 24 h later of the controls, and \(E_0\) and \(E_{24}\); respectively, referring to the areas of the experimental groups at 0 and 24 h.

Odontoblastic differentiation analysis
The odontoblastic differentiation induction medium was prepared as before. Cells were cultured in the induction medium containing different doses (5%, 10%, 20%, 50%, or 80%) of CGF extracts, with the group without CGF supplement taken as the control.

Cellular alkaline phosphatase activity. After induction for 5 and 9 days, the activity of alkaline phosphatase (ALP) was measured using the Alkaline Phosphatase Assay Kit according to the manufacturer’s instructions (Sigma-Aldrich). The absorbances were detected at OD = 520 nm, and the values of enzyme activity were expressed in U/gprot.

Alizarin Red S staining. After 14 and 21 days of induction, cells were stained by 40 mM Alizarin Red S solution (Sigma-Aldrich; pH 4.2) to indicate the extracellular mineral deposits. For semi-quantitative analysis, 10% cetylpyridinium chloride (in 10 mM sodium phosphate, pH 7.0) was added to destrain the Alizarin Red-positive deposition. The absorbance was measured at OD = 562 nm. Values of absorbance in the experimental groups were accordingly converted to a percentage relative to that of the control group.

Expression of odontoblastic characteristics. Protein samples and total RNAs were, respectively, extracted in accordance with the standard protocols. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and western blot analysis were performed as we described previously. Primer sequences of dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are provided in Table 1. The protein expression of DMP-1 (1:4000; Abcam) and DSPP (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) were also detected. β-Actin (1:8000; Santa Cruz) was used as an internal reference. Quantitative data for protein expression were evaluated based on the intensity of protein bands.

Endothelial differentiation analysis
Confluent DPSCs at passage 3 were exposed to the endothelial induction medium for 7 days containing 2% FBS and different concentrations (0%, 5%, 10%, 20%, 50%, or 80%) of CGF, with or without 50 ng/mL of rhVEGF165 (R&D systems, Minneapolis, MN, USA; 293-VE-010/CF). Undifferentiated cells were maintained in DMEM containing 2% FBS and different concentrations (0%, 5%, 10%, 20%, 50%, or 80%) of CGF, with or without 50 ng/mL of of rhVEGF165 (R&D systems, Minneapolis, MN, USA; 293-VE-010/CF). Undifferentiated cells were maintained in DMEM containing 2% FBS as the negative control. The medium was refreshed every other day.

In vitro tube formation assay. Growth factor–reduced Matrigel matrix (Corning Inc., Corning, NY, USA) was added into a cold 24-well plate with 200 μL in each well and incubated at 37°C for 30 min. DPSC-derived ECs were harvested and gently seeded on Matrigel at a density of 85,000 cells per well. At 14 h post seeding, cells were stained by incubating with 2 μg/mL of Calcein AM (Invitrogen, Carlsbad, CA, USA) at 37°C for 30 min. A vessel-like tube network formed by human umbilical vein endothelial cells (HUVECs) was employed as the positive control. The tube-like structure was photographed under a fluorescent microscope (Leica). Levels of tube formation among the VEGF+ groups were evaluated based on the tubular network skeletons identified by ImageJ software. The parameters including the number of nodes and meshes and the total length of segments were calculated.
Expression of endothelial markers. The protein levels of vascular endothelial growth factor receptor-2 (VEGFR2, 1:3000; R&D systems) and cluster of differentiation 31 (CD31; 1:5000; Abcam) in each group were examined by western blot as described above.

Statistical analysis
Data were presented as mean values ± standard error (SE). The equal variance between individual groups was confirmed by Brown–Forsythe test, and then the results were analyzed by variance (one-way analysis of variance (ANOVA)) with Tukey’s post hoc test using GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). p values less than 0.05 were considered statistically significant. Experiments were performed in triplicate independently.

Results

CGF scaffolds possessed unique fibrin network and showed great cytocompatibility
After centrifugation, a buffy gel of CGF was obtained (Figure 1(a)). CGF scaffold was a tough fibrin membrane through mechanical compression from its gel (Figure 1(b)). H&E staining revealed the porous nature of CGF fibrin (Figure 1(c)). The surface morphology by SEM indicated that the CGF scaffolds had complex 3D networks composed of closely interwoven fibrin fibers with numerous platelets and leukocytes trapped inside its meshes (Figure 1(d) and (e)). In addition, DPSCs attached well to the scaffold’s surface by sending out cell processes into the pores of CGF. Close cell connections were built with cellular filopodia stretching fully (Figure 1(f) and (g)). These observations confirmed the great biocompatibility of CGF scaffolds.

CGF promoted DPSC proliferation in a dose-dependent manner
After being cultured for 3 days, more EdU-labeled cells were detected at high doses (50% and 80%) of CGF groups (Figure 2(a)). And the ratios of EdU-positive cells increased significantly with the increase of the concentrations (p < 0.05), exhibiting a dose–response effect (Figure 2(b)).

Cell proliferation rates were tested by CCK-8 assay at different time courses. On the third day, all groups had significant increases in cell number. In the later period of treatment (from day 5 to day 7), cells cultured with high-dosage CGF displayed higher cell proliferation rates. But the viability of cells in the control group which were treated by blank DMEM decreased gradually, which might be due to the absence of nutrition (Figure 2(c)).

The chemotactic activity of CGF on DPSCs
After 24-h treatment, the chemotactic activity of CGF on DPSCs was assessed by in vitro wound-healing assay. The results indicated that the low concentrations (10% and 20%) of CGF significantly stimulated cell migration compared to the controls (p < 0.05). With the relative migration rate 2.88-fold greater than in the control group, 20% CGF displayed the most obvious promotion effect on cell migration. However, a gradual decline was seen with the concentration of up to 50% (Figure 3(b)).

Effects of CGF on mineralization formation and odontoblastic differentiation of DPSCs
After 14 and 21 days of induction, mineral nodules were detected by Alizarin Red S staining (Figure 4(a)). Based on absorbance evaluation, the mineralization levels on day 21 increased dose-dependently in CGF-treated cells and reached a peak at the concentration of 20%. However, the calcium contents in the 50% and 80% CGF groups had no significant difference compared to the controls (Figure 4(b)). The ALP activity and the expression of DSP and DMP-1 were detected for cell differentiation efficiency comparison. We found that, compared to the controls, the 20% CGF treatment significantly upregulated the cellular enzyme activity on day 9 (Figure 4(c)) and the expression of DSP and DMP-1 at both gene (Figure 4(d)) and protein levels (Figure 4(e) and (f)).

The endothelial differentiation–inducing effects of CGF on DPSCs
Capillary-like network formation capacity on Matrigel is a marker of ECs’ function.22 Our results showed that the DPSC-derived ECs in the VEGF-supplemented group formed mature tube-like networks on Matrigel, which were similar to the typical tube structures formed by HUVECs (Figure 5(a)–(e)). Evaluation of the numbers of nodes and meshes and the total length of segments revealed that the DPSC-derived ECs co-induced by VEGF and low-dosage CGF (5% and 10%) had higher tube formation rates than the cells treated only with VEGF (Figure 5(m)). Also, western blot results confirmed it by the significantly upregulated expression of CD31 and VEGFR2, two marker proteins of ECs, in the DPSC-derived ECs concomitantly induced by VEGF and 5% CGF (Figure 5(n)). However, the continued increasing concentrations of CGF led to a reduction in tube formation. The DPSC-derived ECs induced with VEGF and 80% CGF displayed poor tube formation ability on Matrigel (Figure 5(f)).

The ECs’ differentiation capacity of DPSCs induced by CGF-conditioned medium was also detected. A small amount of broken and weak tube structures was formed by 10%, 20%, and 50% CGF-induced cells (Figure 5(i)–(k)). Nevertheless, DPSCs treated with too low or too high
concentrations of CGF (5% or 80%) formed no vessel-like structure on Matrigel (Figure 5(h) and (l)), similar to undifferentiated cells (Figure 5(g)). The expression levels of CD31 and VEGFR2 were higher in the CGF-induced cells, but the difference had no statistical significance (Figure 5(n)).

**Discussion**

Complete dentin–pulp complex regeneration expects a fully functional pulp formation with a balance between the soft and mineralized tissue reconstruction. Efforts have been made in this aspect by examining biological scaffold. In this study, we confirmed that CGF promoted cell proliferation, migration, and the DPSC-mediated dentinogenesis and angiogenesis process.

Autologous platelet concentrations including PRP, PRF, and CGF have been utilized as scaffolds for tissue regeneration. But the application of PRP has a potential risk of immunological rejection and pathogen transmission due to the requirement of anticoagulant and thrombin during preparation. As a modified generation of PRF, CGF is...
Figure 2. The proliferation activity of DPSCs after being treated by different doses of CGF-conditioned medium. (a) The fluorescence images of EdU incorporation assay showed that the proliferative cells were more frequently detected as the concentrations of CGF increased. Scale bar = 100 μm. (b) Comparative analysis of the ratios of EdU-positive cells among all the groups (n = 3). (c) CCK-8 assay indicated that the number of DPSCs increased in the time course, especially those treated by high-dosage CGF (n = 5).

DPSCs: dental pulp stem cells; CGF: concentrated growth factor; CCK-8: Cell Counting Kit-8; EdU: 5-ethyl-2′-deoxyuridine; FBS: fetal bovine serum; NS: not statistically significant.*p < 0.05; **p < 0.01; ***p < 0.001.

Figure 3. The chemotactic activity of CGF extracts on DPSCs. (a) The representative images of wound-healing assay. Scale bar = 200 μm. (b) Relative cell migration rate in each group after 24 h was calculated based on the changes of scratch areas. CGF at low concentrations significantly promoted cell migration when compared with the control group, and 20% (v/v) CGF exerted the optimal effect, while 50% and 80% of CGF showed no significant effect on cell migration.

DPSCs: dental pulp stem cells; CGF: concentrated growth factor; NS: not statistically significant.*p < 0.05; **p < 0.01; ***p < 0.001.
activated via intrinsic coagulation reaction. The staged centrifugal procedure of CGF distinguishes it from PRF. CGF clots have closely interwoven fibers exhibiting a relatively stiffer texture than PRF. A “cocktail” of growth factors were highly concentrated within CGF such as VEGF, TGF-β, and PDGF, all of which have been reported to be able to regulate the biological behavior of diverse cell types. In addition, platelets and leukocytes trapped in CGF were also able to release some chemokines contributing to cell recruitment as well. The dense fibrin network of CGF not only provided matrix surface for cell adhesion and migration, but also protected those bioactive components from proteolysis. As investigated before, the release of CGF maintained up to nearly 2 weeks with a peak concentration on the fifth day. In this study, we soaked the CGF gel in DMEM for 14 days to totally collect its effective constitution. Particularly, CGF extracts were diluted into low concentrations at 5% and 10% in order to imitate the slow release of growth factors from CGF scaffold in vivo.

Rapid expansion, recruitment, and multi-differentiation of stem cells are essential for response to tissue damage and inflammatory condition. Our results revealed that CGF improved the proliferative potential of DPSCs in a dose-dependent manner and achieved maximal promotion when the concentrations reached 50%. But CGF displayed a pleiotropic effect on cell migration and odontoblastic differentiation, showing promotion effect dose-dependently at low concentrations (≤50%), while high concentrations (50% or 80%) of CGF exerting negligible or no effect. Previously, similar phenomenon was observed in the mineralization process of rat dental pulp cells when induced
Figure 5. The effects of CGF extracts on DPSC-EC differentiation. (a–e) DPSC-derived ECs in VEGF+ induced groups formed extensive and mature capillary-like structures on Matrigel, similar to the tubes formed by HUVECs (inside dotted rectangle), (f) while the cells co-induced by VEGF and 80% (v/v) CGF formed few tube structures. (g–l) Some weak tube structures (orange asterisks) were observed in the CGF-induced groups. Bar = 100 µm. (m) Quantitative analysis of the numbers of nodes and meshes and the total length of segments in the VEGF+ group, indicating that 5% CGF had a synergistic effect with VEGF on cell differentiation. (n) Western blot indicated the upregulated expression of VEGFR2 and CD31 in DPSC-derived ECs.

DPSCs: dental pulp stem cells; EC: endothelial cell; HUVECs: human umbilical vein endothelial cells; VEGFR2: vascular endothelial growth factor receptor-2; NS: not statistically significant.

*p < 0.05; **p < 0.01; ***p < 0.001.
under different concentrations of PRP. Qin et al. suggested that it may be associated with the unsuitable pH of culture medium resulting from containing too much platelets. We thought that the high level of leukocytes, interleukin (IL)-1β, and IL-6 might be responsible for the negative role of high-dosage CGF as well. Even though the definite mechanism is still unclear, low concentrations of CGF exerting the optimal effect perfectly matched its trait of slowly releasing effective constituents, which benefited the application of CGF as scaffolds in vivo.

Angiogenesis plays a crucial role during tissue homeostasis and repair for the supply of nutrition and oxygen. It has been reported that DPSCs with highly angiogenic potential are capable of differentiating into ECs in the presence of VEGF. As a natural reservoir of VEGF and many other pro-angiogenic factors, the effect of CGF on endothelial cell differentiation of DPSCs was never investigated. In this study, we found that a low-dosage CGF at 5% had a synergistic action with VEGF on the DPSC–EC differentiation. However, 80% of CGF conversely repressed this process. Based on previous studies that blocking TGF-β signaling enhanced endothelial differentiation, we hypothesized that the negative role of high-dosage CGF may be associated with the excess content of TGF-β with increasing concentration.

Conclusion
The complex fibrin fiber network and great cytocompatibility of CGF made it suitable for cell adhesion. High-dosage CGF stimulated cell proliferation, while the low doses of CGF promoted cell migration and both odontoblastic and endothelial differentiation of DPSCs. Our investigation provided the in vitro experimental evidences of CGF as a new type of growth factor–rich scaffold in dental pulp tissue engineering.

Declaration of conflicting interests
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