Original Article
Effect of basic fibroblast growth factor on the gingiva-derived mesenchymal stem cells

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Abstract: Gingiva-derived mesenchymal stem cells (GMSCs) have recently been harvested and applied in tissue engineering fields. Basic fibroblast growth factor (bFGF) exerts a significant effect on the proliferation and differentiation potential of many types of stem cells. However, the effects of bFGF on the biologic potentials of GMSCs have not been investigated and reported to date. So this experiment is designed and conducted to investigate the effect of bFGF on GMSCs in terms of proliferation and differentiation potential. Treatment with 10 ng/ml bFGF significantly increased the proliferation. Meanwhile, bFGF also enhanced the adipogenic differentiation of GMSCs. In contrast, bFGF of the same amount reduced the osteogenic differentiation of GMSCs. In conclusion, bFGF increases the proliferation ability and affects the differentiation potency of GMSCs.

Keywords: Basic fibroblast growth factor, gingival-derived mesenchymal stem cells, cell proliferation, cell differentiation

Introduction

Mesenchymal stem cells (MSCs), as multipotent stromal cells, can be differentiated into a variety of cell types including osteoblasts, myocytes, chondrocytes and adipocytes [1-4]. This phenomenon has been documented in specific cells and tissues in living animals and their counterparts growing in tissue culture, like osteocytes, teeth and cartilage [5-8]. Gingiva is a unique tissue attached to the alveolar bone, recognized as a biological mucosal barrier and a distinct component of the oral mucosal immunity. Zhang firstly isolated a population of MSCs from gingival tissues that showed stemness. Gingival-derived mesenchymal stem cells (GMSCs) possess more advantages compared to the other dental stem cells. They are much easier to get from the clinically resected gingival tissues. Furthermore, GMSCs are a type of pluripotent stem cells with self-renewal, multipotent differentiation potential and immunomodulatory capacities [9-10]. In addition, with high proliferation capacity, uniformly homogenous property, stable phenotype, and importantly, maintaining normal karyotype and telomerase activity during prolonged culture time, GMSCs are considered as an ideal candidate cell resource for stem cell transplantation, tissue engineering and regeneration nowadays [7, 9-10].

Basic fibroblast growth factor, also known as bFGF, FGF2 or FGF-β, is a member of the fibroblast growth factor family, and its function is mediated through high-affinity receptors [11]. In normal tissue, bFGF is present in basement membranes and in the sub-endothelial extracellular matrix of blood vessels. It stays membrane-bound as long as there is no signal peptide. It has been hypothesized that, during both wound healing and tumor development, the action of heparan sulfate-degrading enzymes activates bFGF, thus mediating the formation of new blood vessels, a process known as angiogenesis. In addition, it is synthesized and secreted by human adipocytes and the concentration of bFGF correlates with the BMI in blood samples [12-13]. In addition, bFGF has been found to act as a mitogen and chemo-attract-
tant that enhances angiogenesis, migration and adipogenic differentiation of both ASCs and BMSCs [14-17].

In dental research, bFGF was reported to enhance the proliferation of human periodontal ligament (PDL) cells in beagle dogs [18-19]. Furthermore, bFGF can exert a significant effect on the proliferation of human dental pulp stem cells (hDPCs) in vitro, while it dramatically induces the mRNA expression of dentin sialophosphoprotein (DSPP) and bone sialoprotein (BSP) in immature adult rat incisor dental pulp cells, suppresses alkaline phosphatase (ALP) activity and osteogenesis differentiation in human dental pulp cells (hDPCs) [20].

However, the effects of bFGF on the biologic potentials of GMSCs have not been investigated and reported to date. The purpose of this study is therefore to isolate GMSCs and culture the GMSCs by adding bFGF in vitro and examine the differentiation changes. Moreover, the responses of GMSCs to various concentrations of bFGF was investigated in terms of proliferation, stem cell marker expression and differentiation potentials.

Materials and Methods

Sample collection, cell culture and single-cell clone of GMSCs

Human gingival tissues were collected from healthy gingiva without any periodontal diseases. Gingival tissues were treated aseptically and incubated overnight at 4°C with Dispase treatment (2 mg/ml; Sigma-Aldrich) to separate the epithelial tissue and spinous layer. The tissues were minced into small fragments (1 to 3 mm²) and digested at 37°C for 45 min in sterile PBS (containing 3 mg/mL collagenase type I Sigma, USA and 4 mg/mL dispase, Sigma, USA). The dissociated cell suspension was filtered through a 70 μm cell strainer (Falcon), and plated on non-treated 10 cm Petridishes (VWR Scientific Products) with complete α-MEM (Invitrogen, California, USA) containing 15% FBS (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 2 mM L-glutamine, and cultured at 37°C in a humidified tissue culture incubator with 5% CO₂ and 95% O₂. The culture medium was refreshed every 3 days. The plastic-adherent confluent cells were passaged with 0.05% trypsin containing 1 mM EDTA and continuously sub-cultured and maintained in the complete growth medium.

A serial dilution method was used to generate single-cell clone culture. The cell suspensions were diluted such that each well of the 96-well plate was seeded with approximately 1 cell. When cells reached confluence of 70-80%, they were transferred to 24-well dishes, and further expanded in the complete growth medium [21]. The experiments were carried out with the working cells of the third passage.

Colony forming units-fibroblast (CFU-F) assay

The CFU-F assay was performed as previously described [22-23]. 3 mL GMSCs of 3rd passage were seeded at 2×10⁴ cells/cm² into 60 mm petridishes with standard medium for 24 hours. The medium was then replaced by fresh medium with the presence or absence of 10 ng/ml bFGF. After 14 days, colonies were washed twice with PBS, fixed for 5 min with 100% methanol, stained with 1% aqueous crystal violet, and counted. A CFU-F was defined as a group of at least 50 cells. The CFU-F assay was repeated in five independent experiments.

Flow cytometric analysis

GMSCs were labeled with fluorescein isothiocyanate-conjugated or phycoerythrin-conjugated antibodies and analyzed with flow cytometry. Cells from different individuals were mixed in equal proportions. GMSCs were harvested with 0.25% trypsin, and cell aliquots (1.0×10⁶ cells) were incubated for 1 h at room temperature with monoclonal antibodies specific for human CD90, CD105, CD146, CD34, CD45 (BD Biosciences), and Stro-1 (R&D Systems) or isotype-matched control IgGs (Southern Biotechnology Associates). Expression profiles were analyzed by flow cytometry (Calibur; BD Biosciences).

As described above, GMSCs from the 3rd passage were grown in standard medium with or without the presence of recombinant human bFGF (10 ng/ml) (PeproTech Inc, Rocky Hill, NJ). After 5 days, the percentages of Stro-1 positive cells were determined by flow cytometry.

Cell counting kit-8 (CCK-8) assay

Various concentrations of bFGF were tested (0, 0.5, 1, 5, 10, and 20 ng/ml) to determine the optimal effect on cell viability. GMSCs (passage 3) were seeded at a density of 1×10⁴ cells/well in 96-well plates. After 24 hours, culture medium was replaced with fresh medium containing...
the above indicated concentrations of bFGF. At different time after treatment, a cell counting kit solution (Cell Counting kit-8, Dojindo, Japan) was added to each well of the plate, and absorbance was measured at 450 nm, according to the manufacturer’s protocol (Dojindo, Japan). For each bFGF concentration tested, 5 treated and 5 control wells were analyzed.

Cell growth curves and population doubling time

GMSCs were seeded at a density of 5.0×10^4 cells/well into 6-well plates with standard medium for 24 hours. The medium was then replaced by fresh medium with the presence or absence of 10 ng/ml bFGF. Cells were counted at 3, 5, and 7 days after seeding. The results shown represent the mean values ± s.e.m. of three separate experiments. To assess the population doubling time, cells were counted at 70-80% confluence. Population doubling time was calculated with the formula (t=ln2/ln(Ct/C0)), where t is the doubling time, t is the time between cell counts, and C0 and Ct (in hours) are the initial cell count and the cell count after time t, respectively.

Alkaline phosphatase activity (ALP) assay

GMSCs were seeded at a density of 1.0×10^5 cells/well into 6-well plates with routine medium. Cells were either pretreated or not pretreated by bFGF (10 ng/ml) for 7 days before the culture was replaced with osteogenic/dentinogenic differentiation media with or without bFGF. After being cultured for various time periods, the ALP activity was detected by using an ALP assay kit (JianCheng Co, Nanjing, China) according to the manufacturer’s instructions. The results were measured spectrophotometrically at 520 nm.

Alizarin red staining

To detect mineralization, cells were maintained in the inducing medium for 2 weeks, and then they were fixed with 70% ethanol and stained with 2% Alizarin red (Sigma-Aldrich). To quantitatively measure calcium, Alizarin Red was destained with 10% cetylpyridinium chloride in 10 mM sodium phosphate for 60 min at room temperature. The calcium concentration was determined by measuring absorbance at 562 nm on a multiplate reader and compared to a standard calcium curve prepared in the same solution. The final calcium level in each group was normalized to the total protein concentration, and measured in a duplicate plate.

Oil red O staining

As described above, GMSCs were seeded at a density of 2.0×10^3 cells/well into 6-well plates with routine medium. Cells were either pretreated or not pretreated with bFGF (10 ng/ml) for 7 days before the culture was replaced with adipogenic induction medium supplemented with 10 μM human insulin, 1 μM dexamethasone, 200 μM indomethacin, and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), with or without bFGF. GMSCs were grown in the adipose-inducing media for 3 weeks. Then, cells were fixed with 10% formalin for at least 1 h at room temperature. Finally, cells were stained with working solution of the Oil Red O for 10 min. The proportion of Oil Red O-positive cells was determined by counting cells [24].

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The expression of adipogenic and osteogenic/dentinogenic markers, including lipoprotein lipase (LPL), peroxisome proliferator-activated receptor gamma (PPARγ) osteocalcin (OCN), bone sialoprotein (BSP) and alkaline phosphatase (ALP), were detected by qRT-PCR. GMSCs were cultured in adipogenic and osteogenic differentiation medium with or without the presence of bFGF. 2 μg aliquots of RNA were synthesized with random hexamers and reverse transcriptase, according to the manufacturer’s protocol (Invitrogen). Real-time PCR was performed with the SYBR Green PCR kit (Qiagen, Germany) and an Icycler iQMulti-color, Real-time PCR Detection System. The gene-specific primer sequences were described as follows: lipoprotein lipase (LPL) forward primer, 5’-CTGGTCGAACATTGGAAT-3’ and reverse primer, 5’-TGTTAGGCATCTGAGA ACGAG-3’; peroxisome proliferator-activated receptor gamma (PPARγ) forward primer, 5’-TCAGTGCCCA GAGGCACCTAC-3’ and reverse primer, 5’-TTCTAGGTCTGTCATTTTCTGGAG-3’; osteocalcin forward primer, 5’-TAAAGAGACCCAGGvCGCTA-3’ and reverse primer, 5’-GATGTGGTCAGCC-3’; Bone Sialoprotein (BSP) forward primer, 5’-TCGAGTCATGCTTCTTGAG-3’; and Alkaline phosphatase (ALP) forward primer, 5’-CTCCTCTTCTTCTC-3’ and reverse primer, 5’-CTCCTCTTCTTCT-3’.
primer, 5'-GGACCATTCCCACGTCTTCAC-3' and reverse primer, 3'-CCTTGTAGCCAGGCCCATT-G-5'; (GAPDH) 5'-CGAACCTCTCTGCTCCTCCT-3' and reverse primer, 3'-CATGGTGT-CTGAGCGATGTGG-5'.

**Statistical analysis**

All statistical calculations were performed with SPSSv.13.0 (SPSS Inc, Chicago, IL, USA) statistical software. Statistical significance was
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Isolation and characterization of GMSCs

GMSCs isolated from gingival tissue were capable of forming adherent clonogenic cell clusters, and most of the cells retained fibroblastic spindle shape (Figure 1A). After 2 weeks of osteogenesis induction, GMSCs formed extensive amounts of ARS-positive mineral deposits (Figure 1D), indicating calcium accumulation in vitro. In addition, some GMSCs developed into oil red O-positive lipid cells after 3 weeks of culture under adipogenic conditions (Figure 1C). GMSCs were positive for the surface markers (STRO-1, CD146, CD105, CD90) but negative for CD34 and CD45 (Figure 1E). Moreover, the results suggested that GMSCs could be steadily expanded in vitro.

Effect of basic FGF on viability of GMSCs

As shown in Figure 2A, bFGF promoted cell proliferation markedly on the 1st, 5th, and 7th day. The dose-response study showed that 10 ng/ml of bFGF had the most optimal effect on cell proliferation, and higher amounts did not increase proliferation consequently. In addition, cell growth curves also showed that bFGF promoted the cell growth on the 3rd and 5th day (Figure 2B). The cell counting assay showed differences between bFGF-treated groups and control groups on the 5th and 7th day (Figure 2A). Moreover, the average doubling time was also different between bFGF-treated groups (24 ± 5.1 h) and control groups (30 ± 6.5 h). These results indicated that bFGF increased the proliferation of GMSCs.

Promotion of basic FGF on colony-forming efficiency of GMSCs

Treatment with bFGF enhanced the colony-forming unit-fibroblastic (CFU-F) forming efficiency of GMSC, as evidenced by the increased number and the increased size of colonies (Figure 3A). The increased percentage of CFU-F formation in the bFGF-treated groups were 5.2~6.8%, which was significantly higher than those in control group (P < 0.05) (Figure 3B).

Effects of bFGF on osteogenic differentiation of GMSCs

ALP assays and ARS were performed to investigate the effects of bFGF on osteogenic differentiation of GMSC. With regarding to ALP, an early marker of osteogenic differentiation, basic FGF suppressed the level of ALP activity in GMSCs (P < 0.05) (Figure 4A). It seemed that mineralization was also affected by bFGF. As shown in Figure 4B-C, GMSCs cultured with osteogenic differentiation medium containing bFGF for 3 weeks led to a decrease in mineralized nodule formation compared with the controls.

Furthermore, real-time RT-PCR results showed lower expression levels of ALP, BSP, and OCN after 3 weeks of osteogenic induction in bFGF-treated groups (Figure 4D).

Effects of bFGF on adipogenic differentiation of GMSCs

After induction with adipogenic medium for 3 weeks, oil red O staining revealed that GMSCs cultured with adipogenic containing bFGF generated larger amount of lipid vacuoles compared with the control (Figure 5A). This was further demonstrated by analyzing the mRNA
levels of PPARγ and LPL. Real-time RT-PCR results showed that PPARγ and LPL expression was enhanced in bFGF-treated group after culturing with adipogenic medium for 3 weeks. The difference was significant ($P < 0.05$) (Figure 5B).

Discussion

Gingival tissue plays an important role by acting as a barrier against different insults, such as chemicals or bacteria, and it exhibits a unique scarless healing process after wounding instead of the scar formation [26], which suggests that some unique types of stem cells may reside in gingival tissues. In the present study, we have isolated and characterized GMSCs, which exhibit several unique stem cell-like properties as MSCs derived from bone marrow and other postnatal tissues [4, 27]. These characteristics include in vitro proliferation as plastic adherent cells with fibroblast-like morphology, colony-forming ability, multi-potent differentiation abilities, and expression of mesenchymal cell surface markers [28]. Moreover, research results have demonstrated that single colony-derived GMSCs possess in vivo self-renewal and differentiation capacities, which deeply supported their stem cell-like properties.

The use of cytokines in patients has been associated with unforeseen adverse events in trials [29]. We must note that the side-effects of cytokines are only beginning to be understood, and their clinical usefulness is therefore hard to predict. Among the cytokines and growth factors, bFGF was clinically proven to accelerate acute and chronic wound healing effectively [30-31]. bFGF has been well known to induce cell proliferation [20, 31-32]. For example, bFGF is critical for the proliferation of hemangiblast, the common progenitor of hematopoietic and endothelial cells, and the proliferation of neural stem cells [33]. It is widely accepted that ESCs require exogenous bFGF to sustain their self-renewal capacity to differentiate into a large number of somatic cells. The present findings in regard to the increased proliferation of GMSCs by bFGF are in agreement with the previously published reports on other MSCs. The present findings indicated that bFGF treatment for 14 days enhanced the cell colony-forming efficiency and also the size of the colonies of GMSCs. Although treatment with bFGF for 28 days did not affect the colony-forming efficiency of bone marrow stromal cells, it did increase the size of colonies [34].

STRO-1 is one kind of antibody that recognizes cell surface antigen present on precursors of various stromal cell types including marrow fibroblasts, osteoblasts, chondrocytes, dipo-cytes, and smooth muscle cells isolated from human adult and fetal bone marrow [35]. Previous studies showed that the expression of STRO-1 in hDPSCs and SCAP was enhanced when cells were cultured in the presence of bFGF [36]. Similar to the reported studies, the GMSCs cultured in the medium containing bFGF expressed STRO-1 more strongly than that without bFGF. GMSCs showed 10.4% positivity for STRO-1 in the presence of bFGF, but only 4.3% in the absence of bFGF.

In the present study, the culture in the presence of bFGF maintained a lower ALP activity and osteogenic differentiation of GMSCs than that in the absence of bFGF. Results of Reverse transcription polymerase chain reaction
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Figure 4. Effects of bFGF on osteogenic differentiation of GMSCs. GMSCs were cultured with osteogenic differentiation medium. A. Cells were either pretreated or not pretreated by bFGF (10 ng/ml) for 7 days. ALP activity was measured, and the untreated group was higher than that in the treated group (**P < 0.01, *P < 0.05). B. After 14 days, cells were stained with 2% Alizarin red (40X). C. Higher mineralization in non-bFGF treated groups after osteogenic induction (*P < 0.05). D. Real-time PCR showed that osteogenic markers, ALP, BSP and OCN, were decreased in the bFGF treated groups.
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(RT-PCR) further indicated that the expression of the osteogenic genes including ALP, osteocalcin (OCN) and BSP was lower in the presence of bFGF. Some reports also have shown that bFGF reduces differentiation and hinders the mineralization of the extracellular matrix in osteoblastic cells, chondrocytes, DPCs, and adipose tissue–derived stromal cells [37]. The result of this study showed that the presence of bFGF decreased mineralization nodes formation, and also suppressed osteogenic differentiation of GMSCs. These findings indicate that bFGF might modulate cell differentiation by suppressing osteogenic differentiation of GMSCs under certain condition.

In addition, opposite to the trend of osteogenic differentiation, GMSCs generated a largest amount of lipid vacuoles in the presence of bFGF. This was further demonstrated by analyzing the mRNA levels of PPARy and LPL, which are adipocyte-specific transcripts. All these findings indicated that GMSCs exhibited strongly adipogenic differentiation potential in the presence of bFGF.

GMSCs constitute more promising alternative to the other dental stem cells in terms of the convenience of isolation, accessible tissue source, and rapid ex vivo expansion. In addition, some recent research reported that GMSCs can function as an immunomodulatory and anti-inflammatory component of the immune system in vivo and are capable of attenuating contact hypersensitivity [10, 38]. Furthermore, with the increasing interest in translational research using GMSCs for the regeneration of tissues, bFGF might be used to maintain stem cell potency of GMSCs in culture, thereby facilitating the long-term usage of cell-based therapies for various tissue regeneration applications, including skin tissue regeneration, vascular formation [39, 40], and even for neural tissue regeneration.

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Disclosure of conflict of interest

None.

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