Acta Odontologica Scandinavica, 2013; 71: 9–14

informa
healthcare

ORIGINAL ARTICLE

bFGF-induced human periodontal ligament fibroblasts proliferation through T-type voltage-dependent calcium channels

DONG-FEI FENG[1], CHUN-YU WANG[2], HAN WANG[3], JING WANG[3], MIAO-MIAO ZHANG[1] & XIAO-HUI JIAO[1]

[1]*School of Stomatology, the First Affiliated Hospital, Harbin Medical University, Harbin, PR China,* [2]*Harbin Children's Hospital, Harbin, PR China, and* [3]*Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University, Harbin, PR China*

Abstract

Objective. To test the hypothesis that T-type voltage-dependent calcium channels (T-CaCNs) are involved in basic fibroblast growth factor (bFGF)-induced proliferation of human periodontal ligament fibroblasts (HPLFs). ***Materials and methods.*** This study examined the expression of the T-type calcium channel sub-units Ca_V 3.1, Ca_V 3.2 and Ca_V 3.3 in HPLFs by real-time PCR. Mibefradil, a T-CaCNs antagonist, was used to block the effect of T-CaCNs and the proliferation of HPLFs was evaluated by the water-soluble tetrazolium (WST) assay. The level of intracellular calcium was measured by laser confocal microscopy. ***Results.*** Expression of the three subunits of T-CaCNs in HPLFs was detected, which was strongly up-regulated upon stimulation by bFGF. The proliferation of HPLFs induced by bFGF was decreased significantly by treatment with Mibefradil. This effect was associated with the decreased expression of T-CaCNs and a decreased level of intracellular calcium. ***Conclusions.*** Expression of the T-CaCNs affected the proliferation of HPLFs that was induced by bFGF, indicating that T-CaCNs could be important in mediating periodontal ligament (PDL) remodeling.

Key Words: *T-type, calcium channels, periodontal ligament, fibroblasts, cell proliferation*

Introduction

During periodontal regeneration, the activity of cells derived from the periodontal ligament (PDL) plays an important role [1] that requires several steps, including recruitment, proliferation and differentiation of PDL cells. The proliferation of PDL cells is usually slow and ineffective after periodontal injury and different methods [2,3] have been used in attempts to increase cell proliferation, including the use of growth factors [4-8]. The basic fibroblast growth factor (bFGF), the most intensively studied among these factors, appears to be effective. bFGF can strongly enhance proliferation of HPLFs and hence periodontal tissue regeneration both *in vitro* and *in vivo* [9-12]. However, details of the underlying mechanism are not available. Full understanding of this mechanism will be helpful to PDL remodeling.

Ca^{2+} signaling is an important growth signal in many cell types. An increased concentration of cytosolic Ca^{2+}, $[Ca^{2+}]_i$, which is controlled by plasmalemmal Ca^{2+} channels, can lead to cell proliferation. Many stimuli have been shown to be able to regulate the activity of the plasmalemmal Ca^{2+} channels [13,14]. There is evidence that T-CaCNs, important components of pasmalemmal Ca^{2+} channels, are associated with cell proliferation, differentiation, growth and death [15-17]; e.g. neuroglioma and vascular smooth muscle proliferation [17-20]. The three T-CaCN sub-units Ca_V 3.1 (α_1G), Ca_V 3.2 (α_1H) and Ca_V 3.3 (α_1I) have been cloned [21,22]. The expression of T-CaCNs changes during development: they are expressed preferentially in fetal and newborn neurons and muscle cells [23] and their expression is decreased during the differentiation of retinoblastoma cells [24]. Because of the lack of specific

Correspondence: Xiao-Hui Jiao, School of Stomatology, the First Affiliated Hospital, Harbin Medical University, Youzheng Street 23, Harbin, PR China. Tel: +86 451 53650087. Fax: +86 451 53625108. E-mail: haerbinjiaoxiaohui@163.com

(Received 25 June 2011; revised 13 September 2011; accepted 27 September 2011)

ISSN 0001-6357 print/ISSN 1502-3850 online © 2013 Informa Healthcare
DOI: 10.3109/00016357.2011.645067

agonists, the inhibitor Mibefradil is commonly used to study the T-CaCNs and it is widely used as a T-CaCN blocker with specific anti-adhesive and anti-proliferative properties. Mibefradil has been shown to be effective in decreasing cell proliferation in diverse cells, such as smooth muscle cells [25].

On the basis of the current knowledge about bFGF and T-CaCN, we asked whether T-CaCN is involved in the bFGF-induced proliferation of HPLFs. The purpose of this study was to investigate the effects of T-CaCN expression on the proliferation of HPLFs induced by bFGF.

Materials and methods

Cell culture

HPLFs, which were donated by Dr Ping Shao of the Dentistry College in Harbin Medical University, were cultured in DMEM with 10% (v/v) fetal bovine serum (FBS, GIBCO, USA), 100 U/ml penicillin and 100 μg/mL streptomycin at 37°C in a 5% (v/v) CO_2 atmosphere. The cells were passaged when the culture flask bottom was ~ 90% confluent. Ninth generation cells were used in this study.

Reagents and experiment design

The inhibitor Mibefradil (Sigma, St. Louis, MO) was dissolved in distilled water at different concentrations (0, 2.5, 5.0 and 10.0 μmol/L) and was added to the cells and growth was continued for 24 h. bFGF (Peprotech Inc., NJ) was dissolved in Tris buffer (5 mmol/L, pH 7.6) at a concentration of 10 ng/mL and was added to the cells and growth was continued for 48 h. Tris buffer or distilled water added to cells without any drug served as controls. We divided HPLFs randomly into the normal cell group and the proliferated cell group. We further divided each group into four sub-groups with different dosages of Mibefradil.

Water-soluble tetrazolium (WST) assay

For the WST cell proliferation assay, 5×10^4 cells were seeded in a 96-well plate with 10 replicates in each group. After treatment of the cells with bFGF and Mibefradil, 10 μL of WST−1 reagent solution (Beyotime, Jiangsu, China) was added to each well and the plates were shaken for 1 min before incubation for 2 h at 37°C in a 5% CO_2 atmosphere. The absorbance at a wavelength of 450 nm was recorded with a Microtiter plate reader (model 680, Boi-Rad, CA) and the data are reported as the percentage of cell survival as compared to the control group.

Real-time PCR assessment of the channel subunits expression

Cells were added to cell culture flasks at a density of 1×10^5/flask and there were five replicates in each group. The sequences of the primers were:

Cav3.1
forward: 5′-CTGTGACCAGGAGTCCACCT-3′
reverse: 5′-TGGGGGCTGAGCGTCTTCAT-3′
Cav3
forward: 5′-GCAACTATGTGCTCTTCAACC-TGC-3′
reverse: 5′-ACACATCTTCAGCTCTGTGGT-CTG-3′
Cav3.3
forward:5′-TGAGCAGCCTTCCAGAGTG-3′
reverse:5′-TCCATGCTCCTCTGAGGC-3′
GAPDH
forward: 5′-GAAGGTGAAGGTCGGAGTC-3′
reverse: 5′-GAAGATGGTGATGGGATTTC-3′

Total RNA from cells was extracted using TRIzol® Reagent (Invitrogen, CA) according to the manufacturer's instructions. Reverse-transcription PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR) were done with the PrimeScript RT reagent kit (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. The PCR program was: 95°C for 30 s followed by 40 cycles of 95°C for 5 s, 55°C for 15 s and 72°C for 10 s, then 72°C for 60 s.

Calcium levels detected by laser confocal microscopy

The cells (1×10^4) were cultured on sterile cover slips in a 6-well plate and there were five replicates in each group. The cells were incubated with Fluo-3/AM (5 μmol/L in DMSO; Sigma) and Pluronic F-127 (0.03% (w/v); Sigma) at 37°C. After 30 min, medium containing Fluo-3/AM was discarded and Hank's solution was added to the adherent cells and incubated for 15 min. Fluorescent changes of the Fluo-3/AM-loaded cells were detected by laser-scanning confocal microscopy (Fluoview-FV300, Olympus, Tokyo, Japan) at a wavelength of 488 nm and a magnification of 4 × 20. The settings of the parameters were: time interval, 5 s; X-point, 43; Y-point, 38; scanning number, 30. Maximum signal, minimum photo bleaching and best signal-to-noise ratio were selected for this study. The fluorescence intensity of images was analyzed by Fluoview-FV1000 software (Olympus).

Statistical analysis of the data

All data were evaluated with a 2 × 4 factorial analysis of variance (ANOVA) design. The SAS 9.1.3 statistical package (SAS Institute, NC) was used to process the data. We compared the data one-to-one by the SNK method. The level of statistical significance was set at $p < 0.05$.

Results

bFGF induces T-CaCN expression and an increased level of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in normal HPLFs

Expression of the sub-units Ca_V 3.1, Ca_V 3.2 and Ca_V 3.3 of T-CaCNs in HPLFs was examined by real-time PCR (Figures 1,2,3). The maximum $[Ca^{2+}]_i$ and the highest level of expression of the three sub-units of T-CaCNs were found in the bFGF-treated cells ($p < 0.05$; Figures 4Ae and B). The expression of Ca_V 3.2 was 4-times greater than that of Ca_V 3.1 and 30-times greater than that of Ca_V 3.1 and Ca_V 3.3.

Mibefradil inhibits the bFGF-induced proliferation of HPLFs

The values in Figure 5 are ratios compared to the growth rate of the normal cell without Mibefradil. When HPLFs were treated with bFGF for 48 h and then Mibefradil was added, cell proliferation was decreased to a level similar to that of the normal cell without Mibefradil. A dose–response inhibition was observed in both the normal cell and the proliferated cell ($p < 0.05$).

The inhibition of bFGF-induced proliferation of HPLFs by Mibefradil is associated with decreased T-CaCN expression and Ca^{2+} level

Proliferated cells treated with Mibefradil showed a significant dose-dependent decreased expression of T-CaCNs compared to bFGF-treated cells (Figures 1,2,3) and the greatest decrease of expression was recorded for the Ca_V 3.2 sub-unit. Decreased $[Ca^{2+}]_i$ was associated with treatment with Mibefradil and bFGF ($p < 0.05$). The greatest decrease of $[Ca^{2+}]_i$ was observed for a Mibefradil dosage of 5.0 μmol/L (Figures 4Ac, Ag and B). Although $[Ca^{2+}]_i$ in HPLFs was increased at a Mibefradil dosage of 10 μmol/L, the difference compared to the normal cell was not significant ($p > 0.05$).

Discussion

Three basic elements must be considered in the design of periodontal tissue engineering; (1) appropriate signals, (2) cells and (3) scaffolds that target the tissue defect [26]. Although many types of cells as well as collagen and bone masses in the organism are deprived when periodontal tissue is damaged, some functional HPLFs and their extracellular matrix remain; i.e. two of the three essential elements, cells and scaffolds, are available. The missing element is a suitable activating signal. It is meaningful to find one effective signal correlated with the proliferation of HPFLs, which plays a crucial role in periodontal regeneration.

T-CaCNs play a very important role in cell growth and proliferation. Normal development tissues, including new endomembrane and proliferating cells, express numerous T-CaCNs. In addition, it has been reported that T-CaCNs are expressed only in actively proliferating cells, whereas L-CaCN is expressed also in cells growing more slowly [16]. We suggest that T-CaCNs are the most relevant to the proliferation of HPDLs. It has been shown that the expression of T-CaCNs sub-units occurs mainly in heart, brain, kidney, skeletal muscle and vascular smooth muscle [20,26]. We examined the expression of T-CaCNs and, to our knowledge, the present study is one of the first to show the presence of T-CaCNs on HPLFs. Further studies of the distribution of T-type calcium ion channel sub-units in other human tissues are needed.

Our results show that all three sub-types of T-type Ca^{2+} channels are expressed in HPLFs,

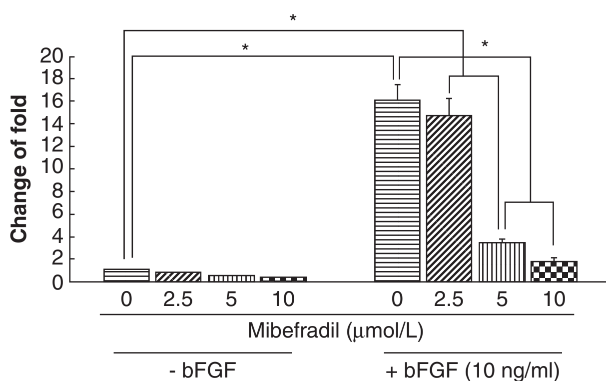

Figure 1. The expression of the Ca_V 3.1 sub-unit of T-CaCNs in HPLFs by real-time PCR ($n = 5$). The highest expression of Ca_V 3.1 was found in the bFGF-only treated cells. Proliferated cells at a dosage of 5.0 and 10.0 μmol/L of Mibefradil showed that expression of Ca_V 3.1 significantly decreased when compared with the bFGF-only treated cells ($p < 0.05$). $^*p < 0.05$.

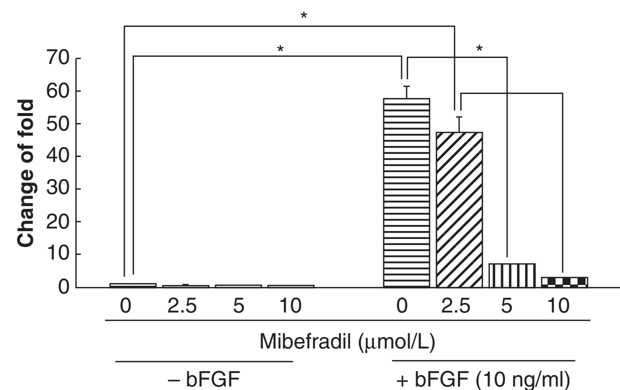

Figure 2. The expression of the Ca_V 3.2 sub-unit of T-CaCNs in HPLFs by real-time PCR ($n = 5$). The highest expression of Ca_V 3.2 was found in the bFGF-only treated cells. Proliferated cells at various doses of Mibefradil showed that expression of Ca_V 3.2 significantly decreased in a dose-dependent manner when compared with the bFGF-only treated cells ($p < 0.05$). $^*p < 0.05$.

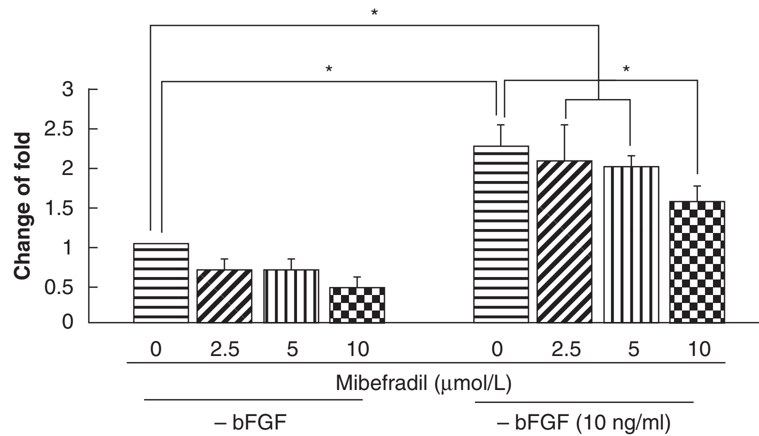

Figure 3. The expression of the Ca_V 3.3 sub-unit of T-CaCNs in HPLFs by real-time PCR ($n = 5$). The highest expression of Ca_V 3.3 was found in the bFGF-only treated cell. Proliferated cells at the highest dose of Mibefradil showed that expression of Ca_V 3.3 significantly decreased when compared with the bFGF-only treated cells ($p < 0.05$). *$p < 0.05$.

whereas, usually, only some of them are expressed in other tissues. In addition, our earlier study showed that the L-type calcium channel is involved also in periodontal tissue rebuilding in HPLFs [27], suggesting an intimate relationship between the repair and regeneration of periodontal tissue and the voltage-dependent calcium channel.

The levels of expression of the three types of T-CaCNs sub-units are not the same. The highest level of expression of Ca_V 3.2 (α_1H) was found in the proliferated cells and was the most sensitive to the blockage effect. Thus, Ca_V 3.2 (α_1H) can be regarded as the predominant action site in HPLFs. To better understand the functions of T-CaCNs in HPLFs, it will be necessary to develop new approaches that are targeted specifically to this class of Ca^{2+} channels and its individual members.

The voltage-dependent calcium channel is a kind of drug receptor. Agonists and antagonists of the calcium channels can increase or decrease the density of the channel [28]. Because there is no report of the concentration of Mibefradil that is effective for the inhibition of proliferation of HPLFs, we used Mibefradil at concentrations of 0–10.0 μmol/L because it has been reported that relatively high concentrations of Mibefradil are selective for T-CaCN and can block it completely [29].

In this study, expression of the three sub-units of T-CaCNs and cell proliferation were increased significantly in bFGF-treated cells. In contrast, after the HPLFs were treated with different concentrations of

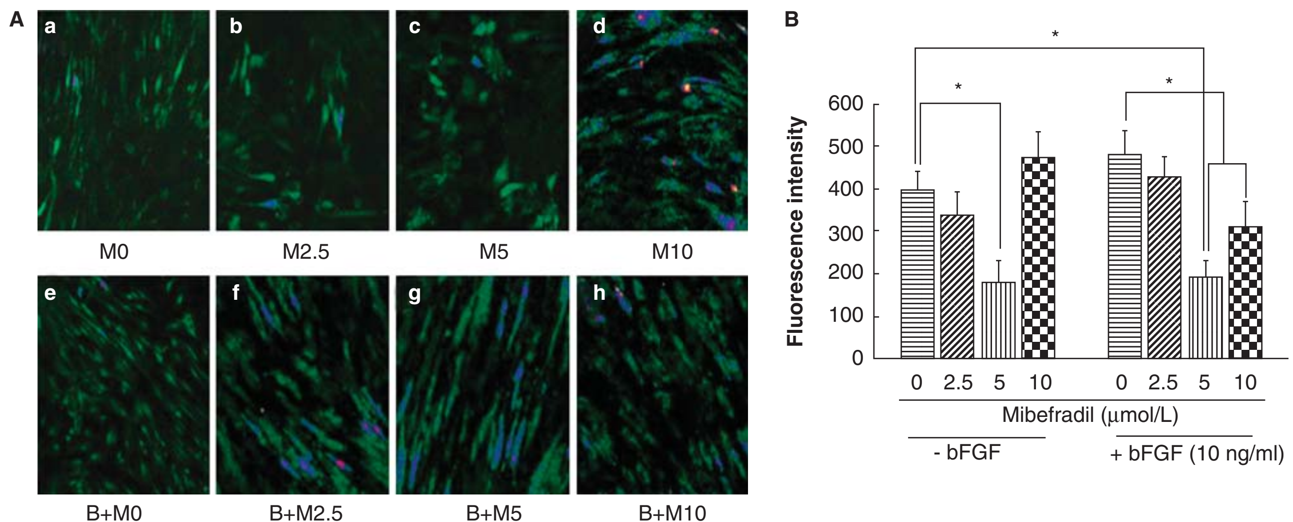

Figure 4. Changes of $[Ca^{2+}]_i$ ($n = 5$). (A) Results of $[Ca^{2+}]_i$ in HPLFs detected by laser confocal microscopy, the changes of $[Ca^{2+}]_i$ are shown in normal cells treated with Miberfradil at doses of 0 (a), 2.5 (b), 5.0 (c) and 10.0 μmol/L (d) as well as proliferated cells treated with Miberfradil at doses of 0 (e), 2.5 (f), 5.0 (g) and 10.0 μmol/L (h). (B) Statistical analysis results of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ decreased significantly in both (c) and (g), there was statistical difference compared to (a) ($p < 0.05$). There was a decreased trend of $[Ca^{2+}]_i$ accompanying Mibefradil treatments with bFGF. $[Ca^{2+}]_i$ decreased to the lowest degree at dosage of 5.0 μmol/L of Mibefradil in the normal cells and bFGF-only treated cells. M represents Miberfradil and B represents bFGF. *$p < 0.05$.

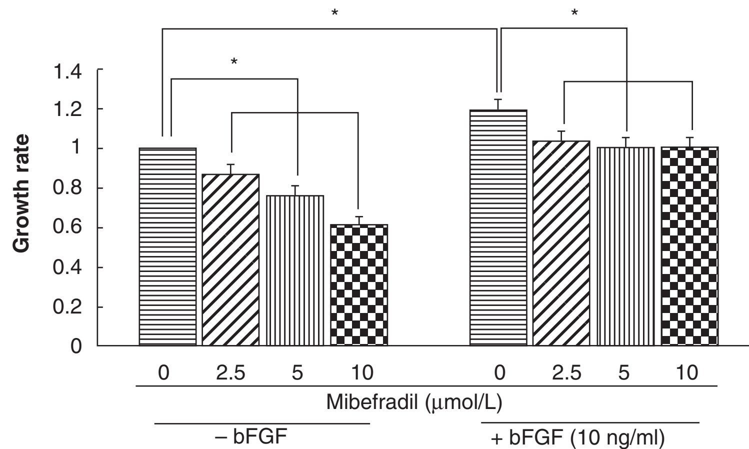

Figure 5. Cell proliferation of HPLFs treated with Mibefradil (n = 10). When HPLFs were treated with bFGF for 48 h and then added Mibefradil, cell proliferation was decreased, to a level which was similar to normal cells without Mibefradil. In both normal cells or proliferated cells, cell inhibition in a dose–response relationship was observed. $^{*}p < 0.05$.

Mibefradil, the expression of T-CaCNs sub-units and cell proliferation showed a dose-dependent decrease in both normal cells and proliferated cells. This suggests a possible mechanism underlying bFGF induction of proliferation of HPLFs is high-level expression of T-CaCNs.

A significant increase of the expression of the three sub-units of T-CaCNs in HPLFs was induced by bFGF, whereas $[Ca^{2+}]_i$ was not increased significantly. This suggests a temporary increase of $[Ca^{2+}]_i$ is an onset signal of cell proliferation, and cells can maintain the $[Ca^{2+}]_i$ level by a self-regulating mechanism. The increase of $[Ca^{2+}]_i$ should be the result of intracellular calcium release because no extraneous calcium was added. In accord with an earlier report [30], intracellular calcium release plays an important role in supporting repeated calcium oscillation by converting cytoplasm into excited medium, which can be a signal for cell mitosis and DNAsynthesis. Concomitantly, the increase of $[Ca^{2+}]_i$ can be decreased by treatment with Mibefradil. In HPLFs, however, 10.0 μmol/L Mibefradil activated Ca^{2+} release from intracellular Ca^{2+} stores, possibly involving IP3 receptors [31,32]. It will be important to understand how Ca^{2+} transients are generated in HPLFs.

Healing of injured or degraded periodontal connective tissues requires migration, proliferation and differentiation of local cells. However, a low proliferative rate of HPLFs in these tissues restricts the extent of healing [33]. If we want to understand the purpose of HPLFs proliferation, perhaps we can investigate the effect of the over-expression of T-CaCNs.

We conclude that T-CaCNs are likely to participate in bFGF-induced proliferation of HPLFs, especially the role of Ca_V 3.2 (α_1H). This effect is associated with an increased level of intracellular calcium. To some extent, our results indicate that calcium channels play an important role in PDL remodeling, which may become a new treatment method for PDL regeneration.

Acknowledgments

The authors thank the laboratory of the Center for Endemic Disease Control of Harbin Medical University for technical assistance.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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