Journal of
PERIODONTAL RESEARCH

J Periodont Res 2013; 48: 740–747

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/jre.12064

C-P. Di[1,2], Y. Sun[1,2], L. Zhao[1,2], L. Li[1,2], C. Ding[1,2], Y. Xu[1,2], Y. Fan[1,3]

[1]Institute of Stomatology, Nanjing Medical University, Nanjing, China, [2]Department of Periodontology, College of Stomatology, Nanjing Medical University, Nanjing, China and [3]Department of Oral Medicine, College of Stomatology, Nanjing Medical University, Nanjing, China

Effect of nifedipine on the expression of keratinocyte growth factor and its receptor in cocultured/monocultured fibroblasts and keratinocytes

Di C-P, Sun Y, Zhao L, Li L, Ding C, Xu Y, Fan Y. Effect of nifedipine on the expression of keratinocyte growth factor and its receptor in cocultured/monocultured fibroblasts and keratinocytes. J Periodont Res 2013; 48: 740–747.

Background and Objective: Keratinocyte growth factor (KGF) and its receptor (KGFR) are involved in hyperplastic diseases. This study explored the effect of intercellular communication on KGF and KGFR in cocultured/monocultured gingival fibroblasts and keratinocytes following treatment with nifedipine.

Material and Methods: Human gingival fibroblasts and keratinocytes were monocultured and cocultured, respectively. MTT was used to investigate the effects of nifedipine on the proliferation of gingival fibroblasts and keratinocytes. Monoculture and coculture systems were treated with different concentrations (0, 0.2 or 20 μg/mL) of nifedipine, and the expression of *KGF* and *KGFR* mRNAs was examined by RT-PCR, whilst the secretion of KGF and the expression of KGFR on the membrane were analyzed using ELISA and flow cytometry, respectively.

Results: Nifedipine (0, 0.2 and 20 μg/mL) had no influence on cell proliferation within 3 d. *KGF* and *KGFR* mRNAs were up-regulated, but only in the cocultures. In coculture, the secretion of KGF was significantly increased by nifedipine, while it was only significantly up-regulated by 20 μg/mL of nifedipine in monoculture. Moreover, the level of KGFR protein in the membrane was significantly increased by 20 μg/mL of nifedipine in monocultures, while it was significantly down-regulated by 20 μg/mL of nifedipine in cocultures.

Conclusion: The expression of KGF and KGFR are influenced by the interplay of gingival keratinocytes and fibroblasts. Epithelial keratinocytes and mesenchymal fibroblasts may interplay to dynamically regulate gene expression, which may have an effect on the gingival condition following treatment with nifedipine.

Yan Xu, DDS, PhD, Department of Periodontology, College of Stomatology, Nanjing Medical University, No.136 Hanzhong Road, Nanjing 210029, China
Tel: 0086 25 85031869
Fax: 0086 25 85031976
e-mail: yanxu@njmu.edu.cn
and
Yuan Fan, DDS, PhD, Department of Oral Medicine, College of Stomatology, Nanjing Medical University, No.136 Hanzhong Road, Nanjing 210029, China
Tel: 0086 25 85031822
Fax: 0086 25 85031976
e-mail: fanyuan65@hotmail.com

Key words: coculture; keratinocyte growth factor; keratinocyte growth factor receptor; nifedipine-induced gingival overgrowth

Accepted for publication January 19, 2013

Nifedipine, a calcium-channel blocker, has been widely used in the treatment of hypertension and/or angina. Gingival overgrowth (GO) is one of the reported side-effects of nifedipine medication, with the incidence ranging from 6.3% to 83% (1–4). GO may compromise esthetics, result in psychological problems and interfere with

normal oral function. Despite the high prevalence and hazards of GO, the pathogenesis has not yet been completely elucidated. It is reported that age and other demographic factors, drugs, concomitant medication, periodontal diseases and genetic factors contribute to the development of GO (5).

An increase in thickness of the epithelium with elongated rete pegs was described following the primal reports of nifedipine-induced GO (1,6,7). However, the pathogenic factors that contribute to the epithelial morphogenesis of this disease are obscure and remain to be studied. It should be taken into account that previous *in vitro* studies investigating the influence of nifedipine were confined to monocultures of gingival fibroblasts or keratinocytes. This simple monoculture system omitted the interactions between the mesenchymal connective tissue and the epithelium. A previous study established, for the first time, cocultures of gingival fibroblasts and gingival keratinocytes, and indicated that gingival fibroblasts were decisive for the manifestation of the phenotype of cyclosporine A-induced GO (8). However, the keratinocytes involved in that study were immortalized with the human papilloma virus type 16 E6/E7 open reading frame, which was gene-modified. Such immortalized cells may compromise exogenous gene expression and phenotype changes and therefore they may not accurately reflect the effects of drugs on native gingival keratinocytes. Therefore, we aimed to establish a coculture system using primary cultures of gingival fibroblasts and keratinocytes.

Among various growth factors that could modulate epithelial cell behavior, keratinocyte growth factor (KGF) is a member of the fibroblast growth factor family, which is produced by cells of mesenchymal origin (9,10) and is known to act in a paracrine manner through its specific receptor, the tyrosine kinase keratinocyte growth factor receptor (KGFR) (11). The KGFR is a splicing variant of the fibroblast growth factor receptor-2 expressed exclusively by epithelial cells (12). The mesenchymal cell–epithelial cell interactions are mediated, in part, by KGF (9). It has been reported that KGF and KGFR are up-regulated in drug-induced gingival hyperplasia (13–15). However, those studies were only related to monocultures *in vitro*.

Therefore, in this study, we compared the effects of nifedipine on the expression of KGF and KGFR in monocultures and cocultures of human primary gingival fibroblasts and keratinocytes, and aimed to explore the effect of intercellular communication on the expression of KGF and KGFR with following treatment with nifedipine.

Material and methods

Cell culture

Normal gingival tissues (without inflammation and overgrowth) were derived from six healthy individuals who were undergoing crown-elongation surgery. Informed consent was received from each participant under a protocol approved by the Ethics Committee of Nanjing Medical University. Gingival fibroblasts and keratinocytes were isolated by tissue-explantation and enzyme-digestion methods, respectively. In brief, the gingival tissue samples were cut into small pieces, placed in a conical tube containing 1 mL of dispase II (2U/mL) and incubated for 18 h at 4°C. The epithelial layers were separated from the connective tissue and then dissociated in 0.25% trypsin/0.01% ethylenediamine tetraacetic acid. The solution was centrifuged to remove trypsin and ethylenediamine tetraacetic acid, and the cells were resuspended in 3 mL of keratinocyte culture medium (Defined K-SFM; Gibco, Grand Island, NY, USA) and seeded into a 60-mm petri dish. The connective tissue was cut into smaller fragments (1 mm×1 mm) for explant cultures and maintained in Dulbecco's modified Eagle's medium containing 10% newborn calf serum and antibiotics (Invitrogen-Gibco, Carlsbad, CA, USA). Gingival keratinocytes at the second or the third passage and gingival fibroblasts between the third and the sixth passage were used in this study.

For cocultures, gingival keratinocytes grown to 80% confluence were trypsinized and then seeded onto the bottom of six-well plates containing removable inserts (Millipore, Bedford, MA, USA) at a cell density of 1×10^5 cells. Gingival fibroblasts were seeded at a cell density of 8×10^4 cells on the inside of the inserts. The devices prevent cross-contamination between gingival fibroblasts and keratinocytes, and thus permit distinct RNA and protein extraction for each cell type.

Nifedipine treatment

A stock solution of nifedipine, of 10 mg/mL, was prepared by dissolving 1 mg of nifedipine (Sigma-Aldrich, St Louis, MO, USA) in 100 μL of dimethylsulfoxide (DMSO) (Amresco, Solon, OH, USA). Taking into account the nifedipine concentrations in the plasma and in the gingival crevicular fluid (16), we chose two concentrations of nifedipine – 0.2 and 20 μg/mL – for use in this study. The final concentration of solvent in the medium was 0.2% (volume by volume). The cytotoxicity assays (MTT and flow cytometry) showed that 0.2% DMSO did not influence cell growth; hence, 0.2% DMSO without nifedipine (0 μg/mL) was used as the control.

Cell-proliferation assay

Cell-proliferation analysis for each group was performed using the MTT assay. This assay is based on the ability of mitochondrial dehydrogenases to oxidize thiazolyl blue (MTT) (Sigma-Aldrich, St Louis, MO, USA), a tetrazolium salt 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide, to an insoluble blue formazan product. In brief, gingival fibroblasts and keratinocytes were seeded in 96-well plates (Corning Life Science, Acton, MA, USA) at relatively low densities (2×10^3 cells/well and 5×10^3 cells/well, respectively). The study groups included different concentrations of nifedipine (0, 0.2 or 20 μg/mL) and four wells were used for each group in each quantitative study. After 1, 2, 3, 4, 5 or 6 d of culture, 20 μL of MTT solution (5 mg/mL

in PBS) was added to each well and incubation was continued for 4 h at 37°C. Then, 150 μL of DMSO was added to each well. The absorbance was determined by eluting the dye with DMSO and the absorbance at 490 nm was measured using a microplate reader (ELX 800; Bio-Tek Instrument, Inc., Winooski, VT, USA). Growth curves were produced of gingival fibroblasts and keratinocytes cultured in all groups.

Regulation of nifedipine on KGF and KGFR expression in monocultures and cocultures

Three groups (0, 0.2 and 20 μg/mL of nifedipine) were considered. After 3 d, total RNA was collected for RT-PCR. Conditioned medium was collected for ELISA. Gingival keratinocytes were also collected for flow cytometry.

RNA extraction and semiquantitative RT-PCR

Total RNA was prepared using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and purity of RNA in each sample were determined by calculating the 260 nm : 280 nm spectrophotometric absorption ratio using the absorption measurements made at 260 nm and at 280 nm (Gene Quant, Piscataway, NJ, USA). One microgram of total RNA per sample was used to generate complementary DNA in a 20-μL reaction mixture (Takara, Otsu, Japan). PCR was carried out using primers specific for the *KGF* gene, the *KGFR* gene and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Table 1), at the same time and under the same PCR conditions. This consisted of repeated cycles of denaturation at 98°C for 10 s, annealing at 57°C for 30 s and extension at 72°C for 30 s. All PCR reactions were carried out within a linear range, using 30 cycles. Ten microlitres of the amplified PCR product from each reaction was electrophoresed in 2% agarose gels. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene, *GAPDH*, the relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with *GAPDH* mRNA was quantified in the photographed gels using a densitometer (Jieda 801; Nanjing Jieda Company, Nanjing, China).

Table 1. Primer sequences of keratinocyte growth factor (*KGF*), keratinocyte growth factor receptor (*KGFR*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)

Gene	Sequence (5′–3′)		Size (bp)	Cycles	Annealing temp. (°C)
KGF	Forward	TCTGTCGAACACAGTGGTACCT	266	30	57
	Reverse	GTGTGTCCATTTAGCTGATGCAT			
KGFR	Forward	CACTCGGGGATAAATAGTTC	150	30	57
	Reverse	CGCTTGCTGTTTTGGCAG			
GAPDH	Forward	GAAGGTGAAGGTCGGAGTC	225	30	57
	Reverse	GAGATGGTGATGGGATTTC			

ELISA

The levels of KGF were determined by ELISA (R&D Systems, Abingdon, UK). Briefly, 10 μL of conditioned medium was directly transferred to the microtest strip wells of the ELISA plate. All further procedures were performed following the manufacturer's instructions. The absorbance at 450 nm was measured using a microtest plate spectrophotometer and the KGF levels were determined using a calibration curve with human KGF as a standard.

Flow cytometry

Gingival keratinocytes were cultured for 3 d in the absence and presence of 0.2 and 20 μg/mL of nifedipine, then detached with 0.25% trypsin/0.01% EDTA in phosphate-buffered saline and centrifuged. Aliquots of 10^5 cells were reacted with the fluorescein isothiocyanate-labeled rabbit anti-human KGFR (BIOS, BoAoSeng, Beijing, China), washed with phosphate-buffered saline and fixed with 1% paraformaldehyde. The average percentage of positive cells, indicating the relative level of KGFR expression, was analyzed using a FACScan flow cytometer (Becton Dickinson, Sandy, UT, USA) and the CELLQuest Software program (CellQuest, Tampa, FL, USA).

Statistical analysis

All assays were repeated three times to ensure reproducibility. Statistical analysis data were expressed as means and standard deviation. The Student's *t*-test was used to evaluate the differences between the control group and the nifedipine group in the MTT assay. The flow cytometry data followed a binomial distribution and an arcsine square-root transformation was performed on the raw data before the analysis. One-way analysis of variance (single factor) was used for statistical evaluation. The Student-Newman–Keuls test was used to compare differences between groups. Values of $p < 0.05$ were considered significant. These analyses were performed using the Statistical Package for Social Science 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Effect of nifedipine on cell proliferation

The effects of 6 d of culture with various concentrations of nifedipine on gingival fibroblasts and gingival keratinocytes were assessed using the MTT assay and the results are shown as growth curves (Figs. 1 and 2). MTT assays showed that during a 5-d incubation period, the 20 μg/mL concentration of nifedipine significantly slowed down the growth of gingival fibroblasts ($p < 0.05$), whilst cell proliferation was slightly enhanced with the 0.2 μg/mL concentration of nifedipine ($p > 0.05$). Neither concentration

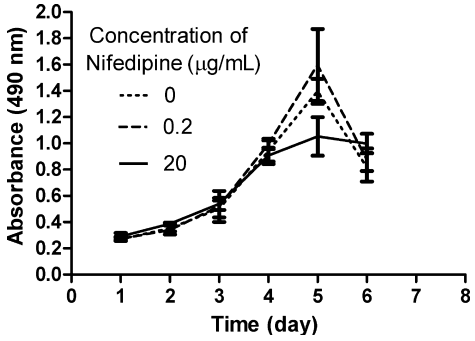

Fig. 1. Growth curves of human gingival fibroblasts cultured with different concentrations (0, 0.2 or 20 μg/mL) of nifedipine. The effect of nifedipine (0, 0.2 and 20 μg/mL) on the proliferation of human gingival fibroblasts was measured using the MTT assay and the experiments were repeated three times. Data are expressed as mean and standard deviation. *Significantly different from the dimethylsulfoxide (DMSO) control at $p < 0.05$ (Student's *t*-test).

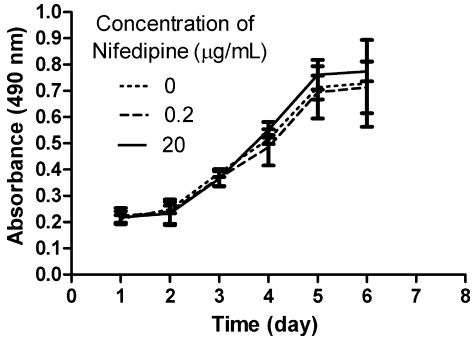

Fig. 2. Growth curves of human gingival keratinocytes cultured with different concentrations (0, 0.2 or 20 μg/mL) of nifedipine. The effect of nifedipine (0, 0.2 or 20 μg/mL) on the proliferation of human gingival keratinocytes was measured using the MTT assay and the experiments were repeated three times. Data are expressed as mean and standard deviation.

of nifedipine had any influence on the proliferation of gingival keratinocytes compared with the control.

Effect of nifedipine on KGF and KGFR expression in monocultures and cocultures

After monoculture with nifedipine (0, 0.2 or 20 μg/mL) for 3 d, the expression of *KGF* and *KGFR* mRNAs showed slight, but not statistically significant, up-regulation (data were not shown). Under the coculture conditions, nifedipine was found to stimulate an increase in expression of *KGF* and *KGFR* mRNAs (Fig. 3A). The relative levels of expression of *KGF* and *KGFR* mRNAs are shown in Fig. 3B. Both 0.2 and 20 μg/mL of nifedipine were found to stimulate a significant increase, of 2.6-fold and 4.1-fold, respectively, in the expression of *KGF* mRNA, compared with the control ($p < 0.05$). Similarly, the expression of *KGFR* mRNA was increased by 4-fold and 11.3-fold, respectively, by 0.2 and 20 μg/mL of nifedipine in comparison with the control ($p < 0.05$).

In addition, the changing trends of expression of KGF and KGFR proteins in cocultures are not consistent with those in monocultures. As shown in Fig. 4, the secretion of KGF was increased by 0.2 μg/mL ($p > 0.05$) and 20 μg/mL of nifedipine ($p < 0.05$) in monocultures of fibroblasts, whereas it was significantly up-regulated by both concentrations of nifedipine in cocultures. The differences in KGFR protein expression on the keratinocyte membrane of cells in the two culture systems are indicated in Fig. 5. In monocultures, the expression of KGFR protein in the group incubated with 0.2 μg/mL of nifedipine showed no difference compared with the control, whereas in the group incubated with 20 μg/mL of nifedipine, it was higher than that of the control ($p < 0.05$) (Fig. 5A and 5C). However, in cocultures, 0.2 μg/mL of nifedipine resulted in a significant up-regulation in the expression of KGFR ($p < 0.05$) whilst 20 μg/mL of nifedipine showed a significant down-regulation in its expression ($p < 0.05$) (Fig. 5B and 5C).

Discussion

The histological findings of nifedipine-induced GO showed fibrosis in the connective tissue, acanthosis of the epithelium and various degrees of inflammatory cell infiltration (17). The effects of nifedipine on gingival fibroblasts have been debated in *in vitro* studies reporting that nifedipine increased or decreased, or did not affect, the proliferation of normal gingival fibroblasts or those isolated from GO. In this study, the results of the MTT test showed that nifedipine (at 0.2 and 20 μg/mL) did not stimulate the proliferation of normal fibroblasts after 3 d of incubation, and that 0.2 μg/mL of nifedipine had, in fact, stimulated a slight increase of cell growth after 5 d of culture. Consistent with our findings, Fujimori *et al.* considered that nifedipine may substantially enhance the proliferation of fibroblasts from healthy human gingiva (18). However, Johnson *et al.* reported that 10^{-7} M nifedipine resulted in a significant decrease in the total number of fibroblasts (19). In addition, some other researchers indicated that nifedipine had no direct effects *in vitro* on the proliferation of gingival fibroblasts from a responder (20). It is believed that this discrepancy results from the different culture systems, target cells, dose and duration of nifedipine treatment.

The majority of research associated with drug-induced GO has focused on factors affecting the gingival fibroblasts, but the gingival epithelium may also be important. Ramon *et al.*

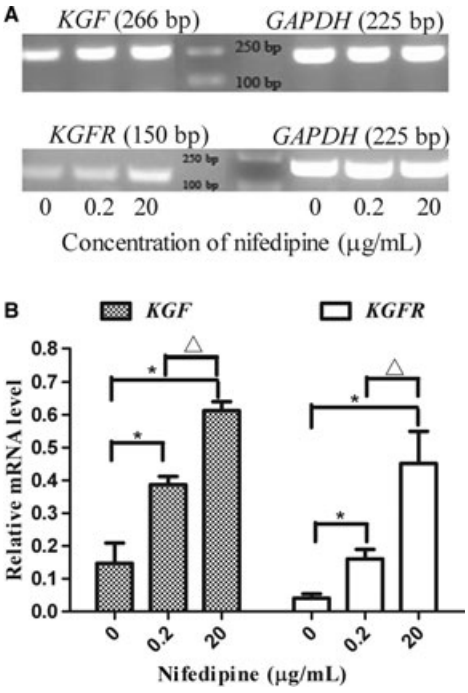

Fig. 3. Expression of keratinocyte growth factor (*KGF*) and keratinocyte growth factor receptor (*KGFR*) mRNAs on the coculture of human gingival fibroblasts and keratinocytes treated with nifedipine. The results were determined using RT-PCR. (A) Effects of 0, 0.2 and 20 μg/mL of nifedipine on the expression of *KGF* and *KGFR* mRNAs in coculture. After coculture with different concentrations of nifedipine for 3 d, total RNA was extracted and used for RT-PCR. A DNA ladder of known base pairs was used for identification of PCR products. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was the housekeeping gene. (B) Levels of expression of *KGF* and *KGFR* mRNAs in the cocultures treated with 0, 0.2 and 20 μg/mL of nifedipine. The relative level was normalized against the level of expression of *GAPDH* mRNA. Triplicate experiments were performed. $*p < 0.05$ compared with the control group (0 μg/mL of nifedipine). $\Delta p < 0.05$ compared with the group treated with 0.2 μg/mL of nifedipine.

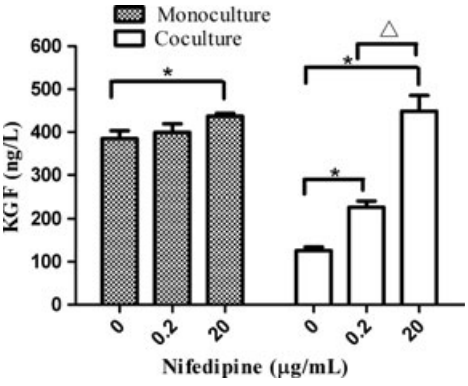

Fig. 4. The changing trends of keratinocyte growth factor (KGF) secretion in monocultures and cocultures of gingival fibroblasts incubated with different concentrations of nifedipine. The amount of KGF secreted in monocultures and cocultures of gingival fibroblasts was measured using ELISA. Monocultures and cocultures of gingival fibroblasts were incubated in the absence (white bars) and presence of 0.2 μg/mL (gray bars) and 20 μg/mL (black bars) of nifedipine. Data are expressed as mean and standard deviation (vertical lines). $*p < 0.05$ compared with the control group (0 μg/mL of nifedipine). $\Delta p < 0.05$ compared with the group treated with 0.2 μg/mL of nifedipine.

showed that the thickness of the oral epithelium in nifedipine-medicated patients was some 5–10 times greater than that of healthy controls (6). However, few studies have investigated the effects of nifedipine on keratinocytes *in vitro*. In the present study, we showed that nifedipine had no obvious influence on the proliferation of gingival keratinocytes *in vitro*. An *in-vivo* study suggested that nifedipine induced epithelial hyperplasia in GO not by an increase in keratinocyte proliferation, but by prolongation of cell life through reduction of apoptosis before epithelial hyperplasia is detectable (21), whereas Nurmenniemi *et al.* concluded that the increased epithelial thickness observed in nifedipine- and cyclosporine A-induced GO was associated with increased mitotic activity, especially in the oral epithelium (22). Although the pathological effects of drugs on the gingival epithelium have been described in those studies, the mechanisms underlying the incrassate epithelium remain inconsistent.

In addition to the direct effects on gingival cells, accumulating evidence indicates an important role of mesenchymal–epithelial interaction in GO development (8,23). The interaction may be regulated through the secreted factors and may thus mediate the process of GO disease, including cell proliferation, differentiation, growth and death. Therefore, the communication between gingival fibroblasts and the covering keratinocytes could not be neglected. Here, we demonstrated that the changing trends of KGF and KGFR expression were different between cocultured and separately cultured systems stimulated with nifedipine.

KGF is synthesized and secreted by mesenchymal cells, such as fibroblasts, and is a potent mitogen for a wide variety of epithelial cells (10). KGF acts via the KGFR, which is expressed only by epithelial cells (12,24). Epithelial cells have been reported to be involved in the epithelial proliferation associated with hyperplastic lesions of various tissues (25–27). Our results were not completely consistent with the studies which suggested that KGF and KGFR were up-regulated in

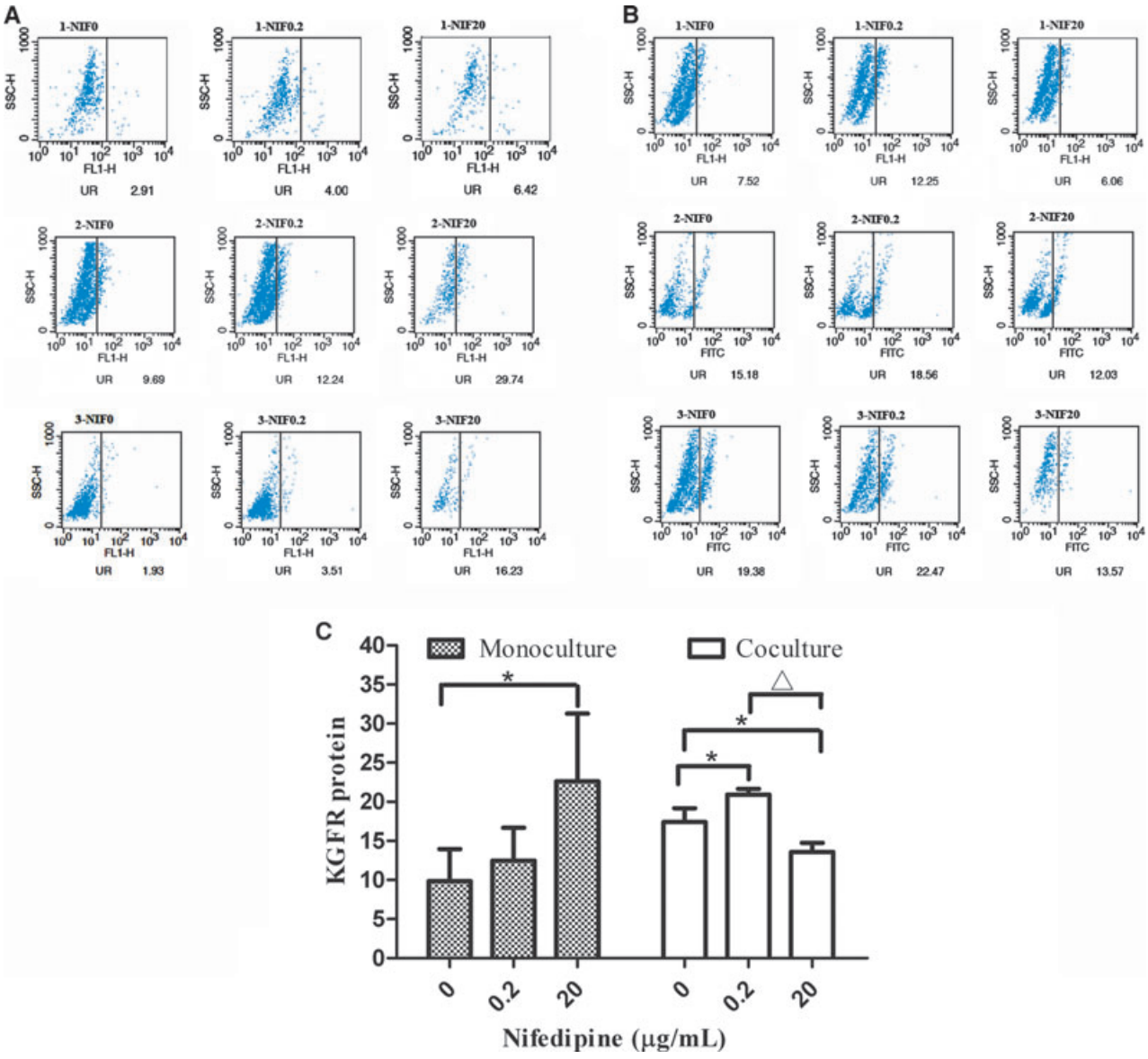

Fig. 5. The changing trends of keratinocyte growth factor receptor (KGFR) expression on the membrane of gingival keratinocytes in monocultures and cocultures treated with different concentrations of nifedipine. (A) The changing trends of KGFR expression on the membrane of gingival keratinocytes in three individual monocultures. (B) The changing trends of KGFR expression on the membrane of gingival keratinocytes in three individual cocultures. (C) Levels of expression of KGFR protein on the membrane of gingival keratinocytes in monocultures and cocultures. Gingival keratinocytes were cultured for 3 d in the absence and presence of 0.2 or 20 μg/mL of nifedipine. UR, mean percentage of positive cells. The mean percentage of positive cells, indicating the relative level of KGFR expression, was analyzed using a FACScan flow cytometer and the CELLQuest Software program. *$p < 0.05$ compared with the control group (0 μg/mL of nifedipine). $\Delta p < 0.05$ compared with the group treated with 0.2 μg/mL of nifedipine.

cyclosporine A-induced gingival hyperplasia and that KGF was up-regulated by the hyperplasia-inducing drug nifedipine *in vivo* and *vitro* (13–15). In our study, similarly to previous studies, the expression of *KGF* and *KGFR* mRNAs was only up-regulated by nifedipine in cocultures. To further investigate the changing trends of KGF and KGFR protein, ELISA and flow cytometry were used to detect the secretion of KGF and KGFR, respectively, on the membrane. Only the highest concentration of nifedipine can significantly promote KGF secretion in monocultures, whereas both concentrations induced significant up-regulation of KGF secretion in cocultures. Interestingly, 20 μg/mL of nifedipine had the opposite effect on the expression of KGFR on the keratinocyte membrane between the two culture systems. The KGFR protein was significantly up-regulated by 20 μg/mL of nifedipine in monocultures, but significantly decreased by it in cocultures. The reason for this contrary result is not clear. It may result from the endocytic pathway, in which KGF and KGFR are transported as active

complexes, first to early and then to late endosomes, and that internalized KGFRs are degraded with slow kinetics (28). Thus, we could speculate that KGFR was functionally down-regulated in cocultures as a result of the interaction between gingival keratinocytes and fibroblasts. Similar studies reported previously that the absence of KGFR protein, despite increased KGFR transcript levels, implied functional receptor down-regulation in the presence of increased KGF in tissue sections from the intermediate phase of wound repair (29). Taken together, compared with the monoculture system, the coculture system is more similar to the *in vivo* conditions and more suitable for use for exploring the mechanisms *in vitro*.

Owing to the lack of culture medium that can support both fibroblasts and keratinocytes, in this study we chose the keratinocyte culture medium (Defined K-SFM) for use in the coculture and used Dulbecco's modified Eagle's medium containing 10% newborn calf serum in the gingival fibroblast monoculture. Despite the fact that the cocultures were serum-free, the fibroblasts had normal shapes and showed significant proliferation after 3 d of incubation. However, gingival fibroblast monolayers could not survive well in the Defined K-SFM for 3 d. This phenomenon further indicated interaction between keratinocytes and fibroblasts in the cocultures. On account of the limits to establish perfect controls, we compared the changing trends of KGF and KGFR, but not the expression levels, in the two culture systems.

In the present study, because KGF was known to act in a paracrine manner through its specific receptor, we only determined the amount of KGF secreted into the medium. Cellular KGF will be further considered so that we can clarify whether nifedipine has any effect on the ultimate production of KGF. This study shows that after treatment with nifedipine the changing patterns of expression of KGF and KGFR in cocultures are not consistent with those in monocultures. The patterns of expression of KGF and KGFR in cocultures treated with nifedipine are more similar to the situation *in vivo*. There may be interplay between epithelial keratinocytes and mesenchymal fibroblasts to dynamically regulate gene expression in order to influence the gingival condition after treatment with nifedipine. The *in vitro* coculture model, which is similar to the *in vivo* situation, can be of great benefit in further studies of the pathological mechanism of gingival hyperplasia.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (Grant No. 81170962), Projects of Science and Technology Department of Jiangsu Province (Grant No. BK2011763 & BK2008363) and Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (Grant No. PAPD2011-2013).

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