Journal of Dental Sciences 17 (2022) 876–881

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Original Article

Enhanced VEGF-A expression and mediated angiogenic differentiation in human gingival fibroblasts by stimulating with TNF-α in vitro

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Received 31 August 2021; Final revision received 13 September 2021
Available online 25 September 2021

KEYWORDS
Angiogenic differentiation;
Human gingival fibroblast;
Tumor necrosis factor-α;
Vascular endothelial growth factor-A

Abstract *Backgroud/purpose:* The effects of inflammatory cytokines were reported to involve in the process of periodontal disease and inflamed tissue enhanced the expression of inflammatory mediators which in turn may promote angiogenesis. Human gingival fibroblasts (HGFs) exert the basic function in periodontal tissue repair and regeneration. However, studies specially focused on the effects of inflammation-related HGFs on angiogenic and osteogenic differentiation are limited. This study was aimed to test whether HGFs enhance vascular endothelial growth factor (VEGF)-A expression mediating angiogenic and osteogenic differentiation by stimulating with tumor necrosis factor-α (TNF-α), to further identify the possible mechanism which may be responsible for this activity.
Materials and methods: In this study, HGFs are treated by TNF-α in order to detect the effects of angiogenic and osteogenic differentiation under inflammation-related condition.
Results: TNF-α enhances VEGF-A expression and results in increasing cell migration and angiogenic differentiation and inhibiting osteogenic differentiation in HGFs. Besides, TNF-α stimulated VEGF-A-mediated angiogenic differentiation is dependent on the activation of mitogen-activated protein kinase (MAPK) pathway, Extracellular signal-regulated kinase (ERK) 1/2 phosphorylation may contribute to regulate the function of VEGF-A in inflammation-related HGFs.
Conclusion: This study demonstrated that enhanced VEGF-A-mediated angiogenic differentiation in HGFs is dependent on the activation of MAPK pathway by stimulating with TNF-α in vitro. Therefore, this study could provide better understand for the progression of inflammation-related periodontal diseases.

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https://doi.org/10.1016/j.jds.2021.09.022

Introduction

Periodontitis is a bacterial infectious disease that many inflammatory cytokines involved in regulating periodontal pathophysiology through crosstalk between tissue cells and periodontal cells.[1,2] Many studies have reported that effects of inflammatory cytokines such as interleukin (IL) −1, IL-6, tumor necrosis factor-α (TNF-α), and inflammation-related cytokine signaling pathways including mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) participated in the process of periodontal diseases.[3,4] It has been reported that increase in the release of IL-1β and TNF-α from peripheral blood mono-nuclear cells of periodontitis patients compared with healthy subjects.[5,6]

During the progression of periodontal disease, the periodontal vasculature is profoundly affected. There was evidence that inflamed tissue enhanced the expression of inflammatory mediators, which in turn may promote angiogenesis. Previous studies suggested that the production of vascular endothelial growth factor (VEGF) was generally upregulated in periodontitis patients.[7,8] From their study suggested that VEGF was likely to be a key factor in the etiology of gingivitis and its progression to periodontitis.[8] As VEGF also could enhance vascular permeability and angiogenesis, it may be related to periodontal disease. However, studies regarding the role of VEGF in the pathogenesis of periodontal diseases still has not been well investigated.

Human gingival fibroblasts (HGFs) are the most abundant cells in gingival connective tissues. The health of gingival tissues depends on the basic functions of HGFs including repair and regeneration. It has been reported that periodontitis is characterized by an imbalance of collagen metabolism, HGF could response to inflammatory cytokines, and further contribute to the progression of periodontitis.[2] Developmental and experimental observations suggested that bone formation could be inhibited by non-ossifying connective tissues.[9] An alternative hypothesis is that fibroblasts constitutively exert inhibitory activity that limits bone growth. It is reported that factors secreted by gingival fibroblasts inhibit BMP-induced osteoblastic differentiation.[10] However, studies specially focused on the effects of inflammation-related HGFs on angiogenic and osteogenic differentiation are limited.

Herein, we utilize TNF-α to stimulate HGFs in order to detect the effects of angiogenic and osteogenic differentiation under inflammation-related condition. Our specific objectives are to test whether HGFs enhance VEGF-A expression mediating angiogenic differentiation and inhibition of osteogenic differentiation by stimulating with TNF-α, to further identify the possible mechanism which may be responsible for this activity.

Materials and methods

Cell culture

Human gingival fibroblast cells (HGFs, HGF-1 cell line, ATCC CRL-2014) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (ScienCell, Carlsbad, CA, USA), and penicillin (100 U/ml)/streptomycin (100 mg/ml) (Gibco). The cells were maintained at 37 °C in a 5% CO_2, 95% humidified atmosphere.

Cell proliferation

The cell proliferation ability was detected by the cell counting kit-8 (CCK-8) assay. HGFs treated with or without TNF-α (10 ng/ml; Sigma–Aldrich, St. Louis, MO, USA) were plated in 96-well plates at an initial density of 2×10^3 cells per well, and then synchronized with serum-free medium for 24 h. For consecutive culturing at 0, 1, 3, 5, 7 and 9 d, the cells were treated with 10% CCK-8 reagent medium solution and incubated at 37 °C for 2 h. The absorbance of samples in triplicate wells was measured with an automatic enzyme-linked immunosorbent assay reader (ELx800, Bio-Tek Instruments Inc., VT, USA) at a wavelength of 450 nm.

Wound-healing assay

For the wound-healing assay, HFGs were allowed to grow to 90% confluence and then wounded by scratching with a pipette tip in the central area. Floating cells and debris were removed, and the medium was changed to serum-free with or without TNF-α. The cells were incubated for 24 h to allow cells to grow and close the wound. Photographs were taken at the same position of the wound at 0 and 24 h time points.

Real-time RT-PCR

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For cDNA synthesis, mRNA was reverse-transcribed into cDNA using the 5 × PrimeScript RT Master Mix (TaKaRa, Kusatsu, Shiga, Japan) at 37 °C for 15 min and 85 °C for 5 s according to the manufacturer's protocol. The synthesized cDNA samples were subjected to determine the expression of VEGF-A, PECAM1, MMP-9, Runx2 and ALP. Gene expression was quantified by Real-Time quantitative PCR using SYBR Green master rox (Roche Diagnostics Ltd, Mannheim, Germany) with a 7500 ABI Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative gene expression was

calculated using the 2 (−ΔΔCT) method. Briefly, the resultant mRNA was normalized to its own GAPDH. The following primers were utilized for the Real-Time RT-PCR. GAPDH (5′-GAAGGTGAAGGTCGGAGTC-3′, 5′-GAGATGGTGATGGGATTTC-3′), VEGF-A (5′-TCACAGGTACAGGGATGAGGACAC-3′, 5′-CAAAGCACAGCAATGTCCTGAAG-3′), PECAM1 (5′-CCTCCAGCCCTAGAAGCCAATTA-3′, 5′-CTCAAAGACTGAGTCAGGCCAGTG-3′), MMP-9 (5′-ACGCACGACGTCTTCCAGTA-3′, 5′-CCACCTGGTTCAACTCACTCC-3′), Runx2 (5′-TCACCTCAGGCATGTCCCTCGGTAT-3′, 5′-TGGCTTCCATCAGCGTCAACACC-3′), ALP (5′- GAGTCGGACGTGTACCGGA-3′, 5′- TGCCACTCCCACATTTGTCAC-3′).

Enzyme-linked immunosorbent assay (ELISA)

VEGF Human ELISA Kit (Abcam, ab100662, Cambridge, MA, USA) is an in vitro enzyme-linked immunosorbent assay, which was performed for the quantitative measurement of VEGF-A in cell culture supernatants. According to the manufacturer's protocol, the conditioned mediums from cells culturing with or without TNF-α were concentrated to 500 μL. Then the samples are pipetted into the wells and VEGF present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-Human VEGF antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of VEGF bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Western blotting

Cells were lysed using a RIPA lysis buffer (Beyotime, Shanghai, China) after treated with TNF-α (10 ng/ml) for 0.5,1 and 2 h. Total protein (30 μg) from each sample was subjected to the SDS-PAGE and then transferred to PVDF membranes (Millipore, Billerica, MA, USA), which were blocked for 2 h at room temperature with 5% nonfat milk in PBST, and then the membranes were incubated with primary antibodies at 4 °C overnight. The following antibodies were used to detect bands on the protein blots: anti-β-actin (1:1000, Abcam), anti-ERK 1/2 (1:500, Abcam), anti-phospho-ERK 1/2 (1:500, Abcam). Then the membranes were incubated with HRP-conjugated secondary antibodies (1:1000, R&D Systems, Minneapolis, MN, USA) for 2 h at room temperature. Immunoreactive material was visualized using the ImmunStar WesternC Kit (Bio-Rad, Hercules, CA, USA) products and bands were detected via Image-QuantLAS4000 (GE, Fairfield, CT, USA). All western bolt analyses were performed at least three independent experiments.

Statistical analysis

The data were representative of three or more independent experiments as the mean ± standard deviation (SD). All the data were normally distributed. Statistical significance was assessed using one-way analysis of variance (ANOVA) and Student's unpaired t-test, using the SPSS 25.0 software package. *P*-value <0.05 was considered significant.

Results

Cell proliferation and migration ability of HGFs by stimulating with TNF-α

The CCK-8 assay was performed to detect the cell proliferation ability of HGFs. After TNF-α (10 ng/ml) stimulation for 0, 1, 3, 5, 7 and 9 d, HGFs displayed no significant difference of cell growth ability than untreated group cells (Fig. 1A). The evaluation of cell migration was performed by wound-healing assay. As shown in Fig. 1B, HGFs treated with TNF-α could close the scratch wound at 24 h while untreated group cells could not. This result indicated that TNF-α could promote migratory ability of HGFs.

Enhanced VEGF-A expression and inhibition of osteogenic differentiation in HGFs by stimulating with TNF-α in vitro

In general, stimulation with TNF-α influenced VEGF-A gene expression in the cultured HGFs. The results of real-time RT-PCR showed that compared with unstimulated HGFs, HGFs stimulated with TNF-α (10 ng/ml) for 24 h, 48 h and 72 h presented significantly increased mRNA levels of angiogenic markers (VEGF-A, PECAM1 and MMP-9) (Fig. 2A—C). Culture supernatants of HGFs unstimulated and stimulated with TNF-α for 24 h, 48 h and 72 h were analyzed for the secreted proteins with ELISA. The results showed that constitutive production of VEGF-A by HGFs treated with TNF-α in vitro (Fig. 2D). Compared with untreated group cells, HGFs treated with TNF-α (10 ng/ml) for 24 h, 48 h and 72 h showed significantly decreased mRNA levels of osteogenic markers (Runx2 and ALP) by real-time RT-PCR (Fig. 2E and F).

Inflammation-related condition activated MAPK signaling pathway in HGFs

To investigate the mechanism of TNF-α induced migration and angiogenic differentiation of HGFs, the activation of MAPK pathway was evaluated in our study. The levels of phosphorylation of Extracellular signal-regulated kinase (ERK) 1/2 in TNF-α-stimulated HGFs were significantly increased in 30 and 60 min (Fig. 3A and B).

To further explore the role of ERK 1/2 in TNF-α-induced migration and angiogenic differentiation, TNF-α-stimulated HGFs were cultured in normal medium containing the ERK 1/2 inhibitor PD98059. Treatment with 20 and 40 μM PD98059 significantly inhibited ERK 1/2 phosphorylation at 30 and 60 min (Fig. 3C and D). Furthermore, PD98059 reduced TNF-α-stimulated cell migration, and significantly inhibited gene expression of VEGF-A, PECAM1 and MMP-9 in HGFs (Fig. 4A—D).

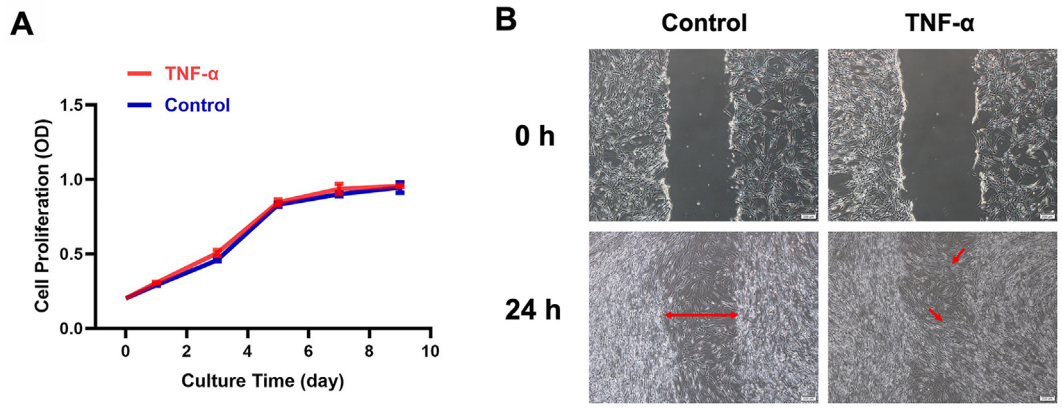

Figure 1 Cell proliferation and migration ability of HGFs by stimulating with TNF-α. (A) Growth curves showed that there was no significant difference of proliferation ability between TNF-α (10 ng/ml) treatment group and nontreatment group in HGFs. The bar represents the mean ± SD. (B) Photographs were taken at the same position of the wound at the indicated time points (× 40 magnification). Bar, 200 μm. The red lines and arrows integrated were indications of the degree of cell wound healing. The wound healing assay indicated that TNF-α could enhance migration behavior of HGFs.

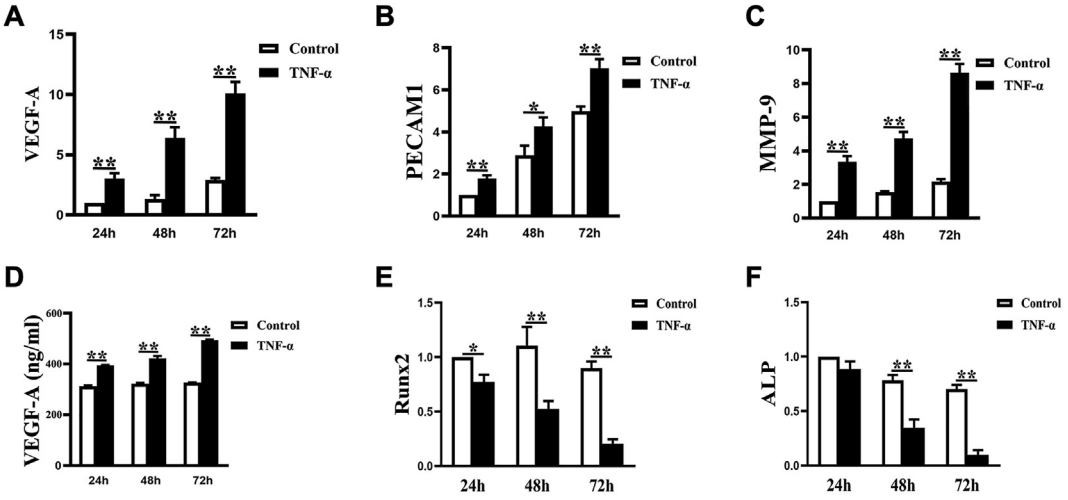

Figure 2 Stimulation of angiogenic differentiation and inhibition of osteogenic differentiation in HGFs by treating with TNF-α in vitro. (A–C) HGFs were treated with TNF-α (10 ng/ml) for 24 h, 48 h and 72 h. Quantitative determination of mRNA expression of angiogenic differentiation marker genes (VEGF-A, PECAM1, MMP-9) were analyzed by Real-Time RT-PCR. GAPDH was used as a control. Each bar represents the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. Data are shown as a fold increase in the mRNA level compared to untreatment group (control). (D) Secreted protein of VEGF-A production from HGFs unstimulated and stimulated with TNF-α for 24 h, 48 h and 72 h were analyzed by ELISA assay. Each bar represents the mean ± SD. $^{**}P < 0.01$. (E and F) HGFs were treated with TNF-α (10 ng/ml) for 24 h, 48 h and 72 h. Quantitative determination of mRNA expression of osteogenic differentiation marker genes (Runx2, ALP) were analyzed by Real-Time RT-PCR. GAPDH was used as a control. Each bar represents the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. Data are shown as a fold increase in the mRNA level compared to untreatment group (control).

Discussion

HGFs participate in immune and inflammatory cascades in periodontal diseases. Inflammatory cytokines in inflamed gingival tissue surround HGFs are essential for the initiation of inflammatory responses.[11] VEGF has the ability to increase vascular permeability, which contributes to increment and extension of inflammation.[12] It has been reported that VEGF is produced by HGF has important implications for the study of periodontal disease and its progression, as well as the healing process that follows periodontal treatment.

In this study, the wound-healing assay results demonstrated that HGFs treated with TNF-α could close the scratch wound at 24 h, while dealing with inhibitor PD98059 reduced TNF-α-stimulated cell migration of HGFs. Furthermore, we found that HGF augmented the production of VEGF-A in response to TNF-α and promoted angiogenic differentiation. Scientists identified VEGF target receptors on endothelial cells and found it to be a key player in physiological and pathological angiogenesis.[13,14] Therefore, we consider the possibility that VEGF-A might be associated with healing of wounds within the periodontal tissue through the promotion of angiogenesis.

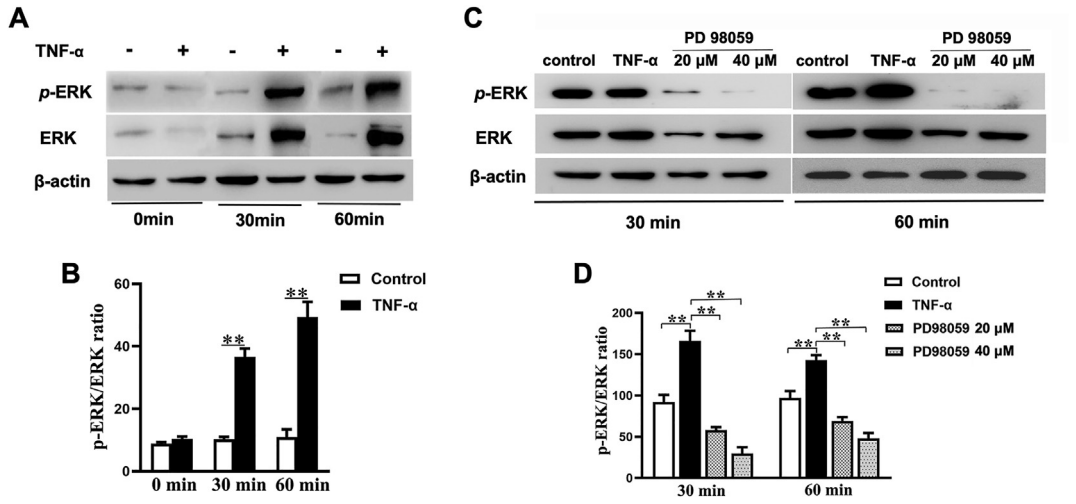

Figure 3 Effects of TNF-α on the activation of the MAPK signaling pathway component ERK 1/2. HGFs were cultured in medium without (control) or with TNF-α (10 ng/ml) for 30 and 60 min. (A) Protein expression (*p*-ERK1/2, ERK1/2) was evaluated by immunoblotting analysis. (B and D) The quantification was performed using image analysis software. Each bar represents the mean ± SD. $^{**}P < 0.01$. (C) Effects of PD98059 (ERK 1/2 inhibitor) on TNF-α-stimulated activation of the MAPK signaling pathway. HGFs were treated with TNF-α (10 ng/ml) for 72 h, and then cultured in medium containing 20, 40 μM PD98059 for 30 and 60 min. Protein expression (*p*-ERK1/2, ERK1/2) was evaluated by immunoblotting analysis.

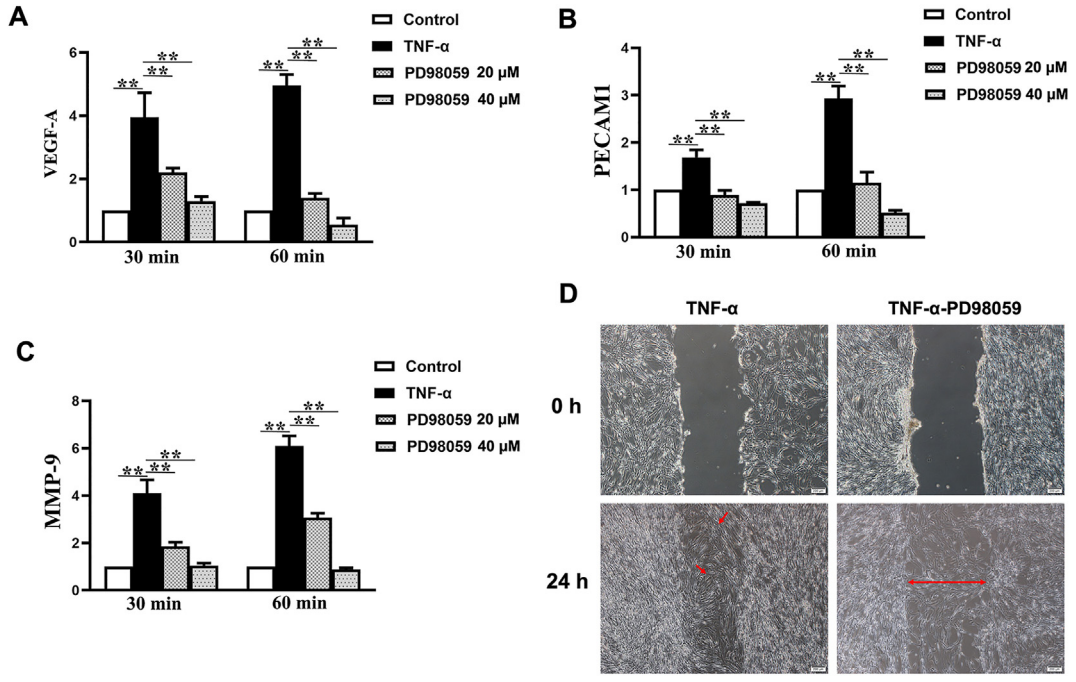

Figure 4 PD98059 reduced TNF-α-stimulated cell migration, and significantly inhibited angiogenic differentiation in HGFs. (A—C) HGFs were treated with TNF-α (10 ng/ml) for 72 h, and then cultured in medium containing 20, 40 μM PD98059 for 30 and 60 min. Quantitative determination of mRNA expression of angiogenic differentiation marker genes (VEGF-A, PECAM1, MMP-9) were analyzed by Real-Time RT-PCR. GAPDH was used as a control. Each bar represents the mean ± SD. $^{**}P < 0.01$. (D) HGFs were treated with TNF-α (10 ng/ml) for 72 h, and then cultured in medium containing 40 μM PD98059 for 24 h. Photographs were taken at the same position of the wound at the indicated time points (× 40 magnification). Bar, 200 μm. The red lines and arrows integrated were indications of the degree of cell wound healing. The wound healing assay indicated that inhibitor PD98059 could reduce TNF-α-stimulated cell migration of HGFs.

The MAPK signaling pathway is composed of a series of serine/threonine kinases that mediates signaling from the cell surface to the nucleus and includes ERK1/2, c-jun N-terminal kinase (JNK), and p38.[15,16] The ERK 1/2 signaling pathway is also significantly involved in cell migration and the secretion of extracellular matrix.[17] Therefore, we

investigated the involvement of the ERK 1/2 signaling pathway in TNF-α treatment. In the present study, phosphorylation of ERK 1/2 was enhanced by TNF-α treatment in HGFs, while inhibitor PD98059 inhibited TNF-α-activated ERK 1/2 signaling pathway, and further reduced VEGF-A expression and mRNA levels of angiogenic markers of HGFs. These results suggested that TNF-α may promote cell migration and angiogenic differentiation via ERK 1/2 signaling pathway in HGFs.

In conclusion, our study demonstrated that TNF-α stimulated VEGF-A expression and results in increasing cell migration and angiogenic differentiation in HGFs. Furthermore, TNF-α stimulated VEGF-A-mediated angiogenic differentiation is dependent on the activation of MAPK pathway, ERK 1/2 phosphorylation may contribute to regulate the function of VEGF-A in inflammation-related HGFs. Collectively, our data may provide better understand for the pathogenesis and progression of periodontal diseases.

Declaration of competing interests

The authors have no conflicts of interest relevant to this article.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant number: 82001104), and the Research Discipline fund from Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, and College of Stomatology, Shanghai Jiao Tong University (grant number: KQYJXK2020).

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