ORIGINAL ARTICLE

Regulatory effects of bone morphogenetic protein-4 on tumour necrosis factor- α -suppressed Runx2 and osteoprotegerin expression in cementoblasts

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Abstract

Objectives: Root resorption is a common phenomenon presented in periodontitis and orthodontic treatment, both of which are accompanied by an elevated TNF- α expression level in the periodontal tissues. Previously, we proved that TNF- α showed an inhibitory effect on cementoblast differentiation, mineralization and proliferation. However, the effect of TNF- α on Runx2 and osteoprotegerin (OPG) expression remains undetermined. This study aimed to identify the influence of TNF- α on Runx2 and OPG expression in cementoblasts and to test whether BMP-2,-4,-6,-7 would affect TNF- α -regulated Runx2 and OPG.

Materials and methods: An immortalized murine cementoblast cell line OCCM-30 was used in this study. The expression of Runx2 and OPG were examined by qRT-PCR after stimulating cells with TNF- α . The role of signalling pathways, including MAPK, PI3K-Akt and NF- κ B, were studied with the use of specific inhibitors. Cells were treated with TNF- α in combination with BMP-2,-4,-6 or -7, then the expression of Runx2 and OPG, the activity of MAPK and NF- κ B pathways, and the proliferation ability were evaluated by qRT-PCR, Western blot and MTS assay respectively.

Results: TNF- α inhibited Runx2 and OPG mRNAs in OCCM-30 cells, and the inhibitory effects were further aggravated by blocking p38 MAPK or NF- κ B pathway. TNF- α -inhibited Runx2 and OPG were up-regulated by BMP-4. The p38 MAPK and Erk1/2 pathways were further activated by the combined treatment of BMP-4 and TNF- α compared with TNF- α alone. Finally, the TNF- α -suppressed proliferation was not obviously affected by BMP-2,-4,-6 or -7.

Conclusions: TNF- α inhibited Runx2 and OPG in cementoblasts, and the p38 MAPK and NF- κ B pathways acted in a negative-feedback way to attenuate the inhibitory effects. TNF- α -inhibited Runx2 and OPG could be effectively up-regulated by BMP-4; however, further investigations are needed to fully elaborate the underlying mechanisms.

1 | INTRODUCTION

Root resorption, which is characterized by the destruction of cementum or even dentin, is a common phenomenon presented in periodontitis^{1,2} and orthodontic treatment.³ In 1996, Lupi et al.⁴ investigated the frequency of orthodontically induced root resorption

based on periapical radiographs, and found that 73% patients suffered from root resorption after orthodontic treatment. Recently, Castro et al.⁵ assessed the frequency of orthodontically induced root resorption again by cone beam computed tomography, and suggested that apical root resorption could be found in all orthodontic patients.

Tumour necrosis factor- α (TNF- α) is a common pro-inflammation cytokine and potent bone loss inducer.⁶ It is mainly secreted by peripheral monocytes,^{7,8} and critically increased in periodontal tissues under periodontitis^{7,9} and orthodontic treatment.^{10–13} Previous study¹⁴ demonstrated that mechanically induced root resorption is nearly completely prevented by systemic application of soluble receptors to TNF- α . These findings suggest that TNF- α may be involved in the pathological process of cementum destruction.

Cementoblasts are the cells located along tooth root surface and are responsible for cementum matrix deposition and mineralization. Besides, cementoblasts also participate in the process of root resorption through crosstalk with cementoclasts.^{15,16} Runx2 is first identified as an osteoblast-specific transcription factor and an essential osteoblast differentiation regulator.¹⁷ In 2009 and 2010, Hirata and Pan's studies revealed that Runx2 is also expressed in cementoblasts¹⁸ and is able to promote the differentiation and mineralization of dental follicle cells via up-regulating cementoblast-related genes, such as bone sialoprotein (BSP), osteopontin, collagen I, cementum protein 23, and cementum attachment protein.^{19,20} Osteoprotegerin (OPG), the decoy receptor for receptor activator of nuclear factor kappa B ligand (RANKL), is a secreted protein that negatively regulates osteoclastogenesis²¹ and also one of the most important cytokines modulating the interaction between cementoblasts and cementoclasts.^{15,16} However, the effects of TNF- α on Runx2 and OPG expression in cementoblasts have not been determined.

Bone morphogenetic proteins (BMPs) are multifunctional glycoproteins which belong to the transforming growth factor superfamily and are actively involved in the development and remodelling of mineral tissues.²² BMPs, such as BMP-2, -4, -6 and -7, have been widely studied in periodontal tissue regeneration experiments because of their osteo-inductive potential.^{23–28} Previous studies indicated that BMP-2 and -6 contribute to the regeneration of cementum,^{26,28} and BMP-4 and -7 promote the proliferation and mineralization of periodontal ligament fibroblasts.^{24,29} Nevertheless, whether BMPs can influence the effects of TNF- α on Runx2 and OPG expression in cementoblasts remains unclear.

In this study, we aimed to clarify the effects of TNF- α on Runx2 and OPG expression in cementoblasts and to test whether BMP-2, -4, -6 and -7 would affect TNF- α -regulated Runx2 and OPG.

2 | MATERIALS AND METHODS

2.1 | Chemicals and antibodies

Recombinant BMP-2, -4, -6 and -7 were purchased from PeproTech (Rocky Hill, NJ, USA). Recombinant murine TNF- α was purchased from Sigma-Aldrich (St Louis, MO, USA). Specific signal pathway inhibitors, including p38 MAPK inhibitor SB203580, Erk1/2 inhibitor PD98059, JNK inhibitor SP600125, PI3K-Akt inhibitor LY294002, and NF- κ B inhibitor pyrrolidine thiocarbamate (PDTTC), were obtained from Sigma-Aldrich. Antibodies for phospho-Erk1/2 (p-Erk1/2, Thr202/Tyr204), total Erk1/2, phospho-p38 MAPK (p-p38, Thr180/Tyr182), total p38 MAPK, phospho-nuclear factor kappaB p65 (p-p65, Ser536) and total

p65 were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2 | Cell culture and treatment

An immortalized murine cementoblast cell line OCCM-30 was provided by Dr. Martha J. Somerman (National Institutes of Health, MD, USA). As previously described,^{30–32} cells were cultured and passaged in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The concentration of FBS was reduced to 5% when TNF- α , BMP-2, -4, -6, -7 or chemical inhibitors were applied. All chemical inhibitors were dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO in the culture medium was no more than 0.1%.³² When cells were treated with chemical inhibitors or BMP-2, -4, -6, -7 in combination with TNF- α , they were added 1 hour before TNF- α treatment. After incubating with TNF- α for indicated time periods, samples were harvested for following experiments.

2.3 | RNA isolation and reverse transcription

Cells were seeded to six-well culture plates (5×10^5 cells/well) and cultured in DMEM containing 10% FBS for 24 hours. Then the culture medium was changed to DMEM containing 5% FBS, and TNF- α , BMPs or chemical inhibitors were applied as indicated. Total RNA was extracted using an E.Z.N.A.[™] HP Total RNA kit purchased from Omega (Norcross, GA, USA). The procedure was performed according to the manual instructions. Purity and concentration were determined using a NanoDrop spectrometer system (NanoDrop Technology, San Diego, CA, USA). cDNA was synthesized using a Thermo Scientific RevertAid First Strand cDNA synthesis kit (Thermo Scientific, Pittsburgh, PA, USA).

2.4 | Quantitative real-time polymerase chain reaction (qRT-PCR)

As previously described,³² qRT-PCR was performed on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). A 20 μ L reaction system containing 1 μ L of cDNA product, 300 nmol/L specific primers and 10 μ L of 2 $\times$ FastStart Universal SYBR Green master mix (Roche, Mannheim, Germany) was applied. Relative mRNA level was calculated using the delta-delta CT method. The sequences of the primers are listed in Table 1.

2.5 | Protein extraction and Western blot analysis

Cells were seeded to 60 mm culture dishes (1×10^6 cells/dish) and then treated with TNF- α or BMPs as described above. Protein was extracted using a total protein lysis buffer (Thermo Scientific) supplemented with protease inhibitor (Roche, Mannheim, Germany). Concentration was measured using BCA method with a kit purchased from Applygen (Beijing, China). After reduction and denaturation, the protein samples were separated through SDS-PAGE and then transferred onto polyvinylidene difluoride membrane.

TABLE 1 Sequences of the primers used for qRT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Runx2	GAATGGCAGCACGCTATTAAATCC	GCCGCTAGAATTCAAAACAGTTGG
OPG	CTGCTGAAGCTGTGGA	GATTTCAGGTCTTTCTCG
Osterix	CCTCTCGACCCGACTGCAGATC	AGCTGCAAGCTCTCTGTAACCATGAC
BSP	GAGCCTCGTGCGACACTTA	AATTCTGACCTCGTAGCCTTCATA
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCTGTTGCTGTA

After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies followed by HRP-conjugated anti-rabbit antibody. The membrane was then visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), and X-ray films were used for signal detection.

2.6 | Enzyme-linked immunosorbent assay (ELISA)

Cells were seeded to six-well culture plates (5×10^5 cells/well) and then treated with TNF- α or BMPs as described above. Cell culture supernatants were collected and used for OPG quantification with an ELISA kit purchased from R&D Systems (Minneapolis, MN, USA). The entire procedure was performed according to the instructions in the manual. The mouse OPG standard provided in the kit was diluted and used for standard curve generation. The plates were determined at wavelengths of 450 nm and 540 nm as recommended.

2.7 | MTS assay

As described previously,³² cells were seeded to 96-well culture plates (5000 cells/well) overnight and then treated with TNF- α in combination with BMPs as indicated. Cell proliferation was evaluated with the CellTiter 96[®] AQ_{ueous} One Solution reagent (Promega, Madison, WI, USA) at indicated time points. The whole procedure was performed

according to the manual instructions, and the plates were read at a wavelength of 490 nm as recommended.

2.8 | Statistical analysis

All experiments in this study were performed three times at least. The qRT-PCR and ELISA data were presented as mean $\pm$ SD of independent experiments. One-way ANOVA was used for significance analysis and $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | TNF- α inhibited Runx2 and OPG mRNAs in OCCM-30 cells

To identify the effects of TNF- α on Runx2 and OPG mRNA expression in OCCM-30 cells, qRT-PCR was performed after incubation with different concentration TNF- α (0–40 ng/mL) for 2 hours (Figure 1A,B). The results demonstrated that both Runx2 and OPG mRNAs were inhibited by TNF- α in a concentration-dependent manner (Figure 1A,B).

To investigate the time-course character, OCCM-30 cells were stimulated with TNF- α (20 ng/mL) for 0–24 hours (Figure 1C,D), and both Runx2 and OPG mRNAs were found to be potently inhibited in a time-dependent manner (Figure 1C,D).

FIGURE 1 Effects of tumour necrosis factor- α (TNF- α) on Runx2 and osteoprotegerin (OPG) mRNA expression in OCCM-30 cells. (A and B) qRT-PCR analysis of Runx2 and OPG mRNA expression after incubating with different concentration TNF- α ranging from 0 to 40 ng/mL for 2 hours. (C and D) qRT-PCR analysis of Runx2 and OPG mRNA expression after incubating with TNF- α (20 ng/mL) for 0–24 hours. * $P < .05$, *** $P < .001$ vs the untreated group

3.2 | Effects of TNF- α on Runx2 and OPG were further aggravated by blocking p38 MAPK or NF- κ B pathway

Previously, we reported that p38 MAPK, Erk1/2, JNK, PI3K-Akt and NF- κ B pathways are strongly activated by TNF- α in OCCM-30 cells.³² We then tried to elucidate the role of the above signalling pathways in the regulation of Runx2 and OPG under TNF- α stimulation.

OCCM-30 cells were pre-incubated with specific chemical inhibitors, including SB203580 (p38 MAPK inhibitor), PD98059 (Erk1/2 inhibitor), SP600125 (JNK inhibitor), LY294002 (PI3K-Akt inhibitor) and PDTC (NF- κ B inhibitor) 1 hour before TNF- α (20 ng/mL) treatment. The concentration of inhibitors was determined according to previous reports^{33,34} and our previous study.³² After incubating with TNF- α for 24 hours, qRT-PCR was performed.

The results demonstrated that none of the inhibitors reversed TNF- α -inhibited Runx2 and OPG mRNA effectively (Figure 2A,B). Interestingly, Runx2 and OPG mRNA became even lower when the p38 MAPK or NF- κ B pathway was blocked (Figure 2A,B). The qRT-PCR data of OPG were reflected in the ELISA measurements, where similar results were obtained (Figure 2C).

3.3 | TNF- α -inhibited Runx2 and OPG were up-regulated by BMP-4

To determine whether BMP-2,-4,-6 and -7 might be useful for promoting cementum regeneration under periodontitis or orthodontic treatment, OCCM-30 cells were pre-incubated with 100 ng/mL BMP-2,-4,-6 or -7 1 hour before TNF- α treatment. The concentration of BMPs was determined according to studies reported previously.³⁵⁻³⁷ qRT-PCR results showed that TNF- α -inhibited Runx2 mRNA was markedly reversed by BMP-2 and BMP-4 (Figure 3A). Similar results were also observed on osterix and BSP (Figure 3B), which are the downstream genes of Runx2.³⁸ OPG expression was determined through both qRT-PCR and ELISA (Figure 3C,D), and both results suggested that BMP-4, but not BMP-2,-6,-7, significantly though not completely eliminated the inhibitory effect of TNF- α on OPG (Figure 3C,D).

3.4 | Effects of BMP-4 on TNF- α -activated p38 MAPK/NF- κ B/Erk1/2 signalling pathways

The above results suggested that the p38 MAPK and NF- κ B pathways exhibit a negative-feedback effect to attenuate the inhibitory effect of TNF- α on Runx2 and OPG. As BMP-4 was shown to be able to rescue TNF- α -inhibited Runx2 and OPG effectively, then we wonder whether BMP-4 could exert its positive effect by modulating the p38 MAPK or NF- κ B signalling pathway.

OCCM-30 cells were pre-incubated with BMP-2,-4,-6 or -7 before TNF- α treatment as described above and then the activities of p38 MAPK, NF- κ B as well as Erk1/2 signalling pathways were examined. Similar to previous study,³² Western blot results demonstrated that p-Erk1/2, p-p38 and p-p65 were all reinforced under TNF- α stimulation (Figure 4A-C). Besides, p-p38 and p-Erk1/2 were further enhanced

FIGURE 2 Roles of MAPK, PI3K-Akt and NF- κ B signalling pathways in tumour necrosis factor-alpha (TNF- α)-regulated Runx2 and osteoprotegerin (OPG) expression. Cells were pre-incubated with SB203580 (10 μ mol/L), PD98059 (10 μ mol/L), SP600125 (10 μ mol/L), LY-294002 (10 μ mol/L) and PDTC (30 μ mol/L) 1 hour before TNF- α treatment. After incubating with TNF- α (20 ng/mL) for 24 hours, qRT-PCR (A and B) was performed to determine the mRNA level of Runx2 and OPG, and ELISA (C) was performed to determine the expression level of OPG. * $P < .05$, ** $P < .01$, *** $P < .001$

markedly by the combined treatment of BMP-4 and TNF- α compared with TNF- α alone (Figure 4A,B). Nevertheless, TNF- α -activated p-p65 was not significantly affected by any of the BMPs used (Figure 4C).

FIGURE 3 Regulatory effects of bone morphogenetic proteins (BMP)-2/-4/-6/-7 on tumour necrosis factor-alpha (TNF- α)-suppressed Runx2 and OPG expression. Cells were pre-incubated with BMP-2/-4/-6/-7 (100 ng/mL) 1 hour before TNF- α treatment; 24 hours after TNF- α (20 ng/mL) treatment, qRT-PCR (A-C) and ELISA (D) were performed to examine the expression of Runx2, osterix, bone sialoprotein (BSP), and OPG. * $P < .05$, ** $P < .01$, *** $P < .001$

FIGURE 4 Effects of bone morphogenetic proteins (BMP)-2/-4/-6/-7 on tumour necrosis factor-alpha (TNF- α)-activated Erk1/2, p38 MAPK and NF- κ B pathways. Cells were pre-incubated with BMP-2/-4/-6/-7 1 hour before TNF- α treatment. After 1 hour of TNF- α incubation, the activities of the Erk1/2 (A), p38 MAPK (B) and NF- κ B (C) signalling pathways were determined via Western blot

3.5 | Effects of BMP-2,-4,-6 and -7 on TNF- α -inhibited proliferation

Previously, we reported that TNF- α exhibited an inhibitory effect on cementoblast proliferation.³² Here, to investigate the regulatory effects of BMP-2,-4,-6 and -7 on TNF- α -inhibited proliferation, OCCM-30 cells were treated with TNF- α in combination with BMP-2,-4,-6 or -7 as indicated (Figure 5). MTS assay revealed that the proliferation of OCCM-30 cells was not obviously affected by any of the above treatment after 24 hours and 48 hours stimulation (Figure 5A,B). In the 72-hour treatment group, the proliferation was significantly suppressed by TNF- α as observed before³² and the TNF- α -suppressed proliferation was not obviously affected by BMP-2,-4,-6 or -7 (Figure 5C).

4 | DISCUSSION

We previously reported that TNF- α impedes the mineralization ability of cementoblasts through inhibiting differentiation and inducing apoptosis;³² however, the effects of TNF- α on Runx2 and OPG expression in cementoblasts remain unclear. In this study, we proved that TNF- α inhibited Runx2 in OCCM-30 cells. This result was similar with the study conducted in osteoblasts,⁶ and provided further evidence that TNF- α inhibited cementoblast differentiation. The effect of TNF- α on RANKL expression was also evaluated, however, reproducible results were not reached, which may be because of the difference in cell generation.

Previous studies conducted by Catrina Al³⁹ and Zhao B⁴⁰ indicated that TNF- α suppressed OPG expression in synovial cells, while

FIGURE 5 Effects of bone morphogenetic proteins (BMP)-2/-4/-6/-7 on tumour necrosis factor-alpha (TNF-α)-inhibited proliferation. Cells were treated with TNF-α (20 ng/mL) in combination with BMP-2/-4/-6/-7 (100 ng/mL) as indicated. After incubation for 24 hours (A) 48 hours (B) and 72 hours (C) the proliferation ability was detected via MTS assay. ** $P < .01$

some other studies suggested that TNF-α increased OPG expression in osteosarcoma MG-63 cells,⁴¹ prostate cancer cells,⁴² rheumatoid synovial fibroblasts,⁴³ mesenchymal stem cells⁴⁴ and melanoma cells.⁴⁵ Our study demonstrated that OPG was inhibited by TNF-α in OCCM-30 cells; therefore, suggested that TNF-α contributes to root resorption not only through inhibiting cementoblast mineralization³² but probably also through regulating cementoblast-mediated cementoclastogenesis. The divergence between the above studies concerning the effect of TNF-α on OPG might attribute to the difference in cell type and the concentration and duration of TNF-α administrated.

In this study, we found that Runx2 and OPG expression inhibited by TNF-α were both markedly rescued by BMP-4. This result is partially supported by Tazoe's study which demonstrated that BMP-4 up-regulates OPG expression in ST2 cells.⁴⁶ The regeneration of cementum is one of the most crucial aspects during periodontal tissue regeneration;⁴⁷ these findings indicate that BMP-4 may be useful for promoting cementum regeneration. Nevertheless, further experiments are still required to observe the effects of BMP-2,-4,-6 and -7 on TNF-α-inhibited mineralization in cementoblasts.

With the use of specific chemical inhibitors, we proved that the p38 MAPK and NF-κB pathways, though activated by TNF-α, acted in a negative-feedback way to the inhibitory action of TNF-α. Further studies revealed that the activity of p38 MAPK, but not NF-κB signalling pathway, was further activated by the combined treatment of BMP-4 and TNF-α compared with TNF-α treatment alone. Similar results concerning the effect of BMP-4 on p38 MAPK pathway were reported in MC3T3-E1 and human mesenchymal stem cells.^{48,49} Tazoe⁴⁶ further proved that BMP-4 up-regulated OPG expression dependent on p38 MAPK pathway in ST2 cells. Based on these results, we speculated that BMP-4 rescued TNF-α-suppressed OPG expression probably by further enhancing the p38 MAPK pathway in cementoblasts.

In summary, we conclude that TNF-α inhibited Runx2 and OPG expression in cementoblasts. The NF-κB and p38 MAPK pathways, though activated by TNF-α, exerted a negative-feedback effect on the inhibitory action of TNF-α. BMP-4 was found to be able to

effectively rescue the TNF-α-suppressed Runx2 and OPG, and this process is probably dependent on the p38 MAPK pathway; however, further studies are still needed to fully elaborate the accurate mechanism.

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CONFLICT OF INTEREST

All authors declare that there is no conflict of interest in this work.

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