Original Paper

Pro-Inflammatory Cytokine TNF- α Attenuates BMP9-Induced Osteo/Odontoblastic Differentiation of the Stem Cells of Dental Apical Papilla (SCAPs)

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Key Words

BMP9 • TNF- α • Periapical periodontitis • Mesenchymal stem cells • Inflammation • Osteogenesis • Dental regeneration • Bone regeneration

Abstract

Background/Aims: Periapical periodontitis is a common oral disease caused by bacterial invasion of the tooth pulp, which usually leads to local release of pro-inflammatory cytokines and osteolytic lesion. This study is intended to examine the effect of TNF- α on BMP9-induced osteogenic differentiation of stem cells of dental apical papilla (SCAPs). **Methods:** Rat model of periapical periodontitis was established. TNF- α expression was assessed. Osteogenic markers and ectopic bone formation in iSCAPs were analyzed upon BMP9 and TNF- α treatment.

Results: Periapical periodontitis was successfully established in rat immature permanent teeth with periapical lesion in which TNF- α was shown to release during the inflammatory phase. BMP9-induced alkaline phosphatase activity, the expression of osteocalcin and osteopontin, and matrix mineralization in iSCAPs were inhibited by TNF- α in a dose-dependent fashion, and high concentration of AdBMP9 partially overcame TNF- α inhibition. Furthermore, high concentration of TNF- α effectively inhibited BMP9-induced ectopic bone formation *in vivo*.

Conclusion: TNF- α plays an important role in periapical bone defect during the inflammatory phase and inhibits BMP9-induced osteoblastic differentiation of iSCAPs, which can be partially reversed by high levels of BMP9. Therefore, BMP9 may be further explored as a potent osteogenic factor to improve osteo/odontogenic differentiation in tooth regeneration in chronic inflammation conditions.

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Introduction

While occurring in all ages periapical periodontitis of deciduous tooth can be caused by caries, tooth trauma or fracture of abnormal central cusp and is one of the most prevalent oral diseases in children. The inflammatory injury of the periapical tissue may lead to bone defects and disturb the root development. Bone defects can aggravate the immature teeth looseness and increase the treatment difficulty. The limitations of current treatments have attracted increased interests in exploring the new treatment methods [1, 2]. In recent years, tissue regenerative engineering has been a focus of tooth tissue repair. Dental stem cells have multiple differentiation potential to regenerate intended tissue types and are the basis of any successful tissue engineering.

The stem cells from apical papilla (SCAPs) are one of the five types of dental stem cells, which are considered as a population of mesenchymal stem cells (MSCs)-like cells. Several studies have demonstrated that SCAPs possess the osteo-/odontogenic potential and researchers believed that SCAPs could be valuable seed cells for the periapical tissue regeneration [3-6]. However, the existing studies were mostly performed under minimal inflammation condition. During the inflammation phase of periapical periodontitis chemokines and pro-inflammatory cytokines are largely released by activated macrophages. Growing evidence suggested that the osteogenic differentiation of MSCs may be regulated by molecular signals from the extracellular microenvironment and the pro-inflammatory cytokines in the cell microenvironment [7-10].

Tumor necrosis factor- α (TNF- α) is one of the largest quantity of pro-inflammatory cytokines during the inflammation phase of periapical periodontitis, which was considered as a major contributor to bone pathophysiology due to its stimulation of bone resorption [11-13]. In mature osteoblasts, TNF- α inhibits the expression of genes necessary for bone formation and stimulates the expression of genes to induce osteoclasts [14, 15]. It was shown that high dose of TNF- α suppresses the osteogenic differentiation of MSC-like cells by decreased levels of alkaline phosphatase (ALP), osteocalcin (OCN), and Runx2 [16-18]. On the other hand, several studies suggested that TNF- α may have an inhibitory effect on BMP-induced osteoblast differentiation of MSCs or preosteoblastic cells [19-21].

BMP9 (also known as growth differentiation factor 2, or GDF2) is one of the most potent factors that can induce osteogenic differentiation of MSCs [22-24]. We have recently established the reversibly immortalized mouse stem cells of apical papilla tissue of mouse lower incisor teeth (iSCAPs), which were shown to retain the ability to differentiate into multiple lineages [25, 26]. We also demonstrated that BMP9 can effectively induce osteo-/odontoblastic differentiation of the iSCAPs [25, 26]. However, considering that tissue regeneration may occur under the inflammation phase of periapical periodontitis, the effect of high dose of TNF- α on BMP9-induced osteogenic differentiation of iSCAPs remains unclear.

In this study, we established a rat model of periodontitis of the immature permanent teeth with periapical lesions to detect TNF- α expression during the inflammatory phase of periodontitis. Furthermore, we investigated the effects of high doses of TNF- α on BMP9-induced osteoblastic differentiation of iSCAPs. Our results demonstrate that TNF- α plays an important role in the induction of periapical bone defect during the inflammatory phase and inhibits BMP9-induced osteoblastic differentiation of iSCAPs in periapical bone regeneration. Therefore, our results strongly suggest that BMP9 may be further explored as a potent osteogenic factor to improve the efficacy of osteoblastic and odontogenic regeneration in tooth engineering under chronic inflammation conditions.

Materials and Methods

A rat model of periapical periodontitis of immature permanent teeth with periapical lesions

The use and care of all animals in this study was approved by the Experimental Animal Ethics Committee of Chongqing Medical University, Chongqing, China. Thirty-five Sprague-Dawley rats (male, 4-week-old)

were purchased from the Laboratory Animal Center of Chongqing Medical University (Chongqing, China) and were randomly divided into seven groups. Animals were anaesthetized by intraperitoneal injection of pentobarbital (30mg/kg). The pulp of maxillary first molar of each rat was exposed to the oral environment. Five rats of each group on days 0 (control), 3, 7, 14, 21, 28, and 35 after lesion induction were sacrificed for microCT and histologic analysis.

Micro-computed tomography (μ CT) analysis

The rat maxillae were retrieved and fixed with 4% paraformaldehyde at 4°C for 48h, and then taken to a uniform position to hold the same angulation to the film [27]. Digital images were taken using μ CT (vivaCT 40, SCANCO) and analyzed the images by calculating the area of mesial root apical lesion using the μ CT software.

Immunohistochemistry

After microCT imaging, the retrieved samples were demineralized with 17% EDTA (pH 7.4) solution for 2 weeks, followed by paraffin embedment. The embedded samples were serially sectioned, deparaffinized, rehydrated and subjected to immunohistochemical staining using the primary antibody (Polyclonal rabbit anti-TNF- α , 1:100; Abcam, UK; no.ab6671) for 24 h at 4°C. The primary antibody was then removed by washing the slides thrice with PBS for 5 min. The sections were incubated with secondary antibody kits (SAB, USA; no.L3012-2) according to the manufacturer's protocol. The sections were stained for 30-40s with diaminobenzidine (DAB) and the nucleus was counterstained with hematoxylin for 10s. Images were obtained using a microscope with a camera (Leica, Germany).

Cell culture, recombinant adenoviruses and chemicals

The iSCAPs which were previously characterized [25], the 293pTP line was used for adenovirus amplification. Both 293pTP and iSCAPs were maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco), 100 units of penicillin and 100 mg of streptomycin at 37°C in 5% CO₂. Recombinant adenoviruses expressing BMP9 and/or GFP were constructed as previously reported [25, 26]. Polybrene (100 μ g/ml) was used to enhance infection efficiency for all adenoviral infections [28]. Recombinant mouse TNF- α was purchased from Sino-Bio (Beijing, China).

Alkaline phosphatase (ALP) staining and quantification assays

The iSCAPs were seeded into 24-well plates and infected with AdBMP9 or AdGFP. The experiment groups were added with 0.1, 1.0, 100 ng/ml TNF- α . Alkaline phosphatase (ALP) staining and quantitative assays were done on day 3 after infection. ALP staining was performed according to the protocol of the NBT/BCIP staining kit (Sino-Bio, China). ALP activity was calculated the absorbance value at 405nm based on the rate of conversion of p-nitrophenyl phosphate disodium salt hexahydrate to p-nitrophenol (Sigma, USA). Each sample was measured in triplicate. ALP activity was normalized by calculating the total protein content of the sample.

RNA isolation, reverse transcription and quantitative real-time PCR (qPCR)

Total RNA was isolated using the Trizol reagent (Invitrogen), and subjected to reverse-transcription using the PrimeScript Reverse Transcription Kit (Takara, Japan). The qPCR analyses were performed in the ABI 7500 Real-Time PCR System with the SYBR Green PCR master mix reagent (Takara) in a 40-cycle PCR cycling program was 95 °C for 30 s, 95 °C for 5 s, 60 °C for 34 s and 95 °C for 15 s. The target gene expression was normalized relative to the level of D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences were: for mouse OPN, 5'-CCT CCC GGT GAA AGT GAC-3' and 5'-CTG TGG CGC AAG GAG ATT-3'; for mouse OCN, 5'-CCT TCA TGT CCA AGC AGG A-3' and 5'-GGC GGT CTT CAA GCC ATA C-3'; and for mouse Gapdh, 5'-ACC CAG AAG ACT GTG GAT GG-3' and 5'-CAC ATT GGG GGT AGG AAC AC-3'.

Alizarin Red S staining

The iSCAPs were seeded into 24-well plates and infected with AdBMP9 or AdGFP. After grown to 80% confluence, cells were incubated in mineralization medium (normal culture medium containing 50 mg/ml ascorbic acid, 10 mM β -glycerophosphate) supplemented with different concentrations of TNF- α . After 2 weeks, the cultures were washed 3 times in PBS and fixed in 4% polyoxymethylene for 30 minutes,

washed with deionized water 3 times, and then stained with 0.4% Alizarin Red S (Sigma-Aldrich, USA) for 30 minutes at room temperature. The excess dye was removed by washing 3 times with deionized water. PBS was added to each well to prevent the cells from drying before the images were acquired.

iSCAPs stem cell implantation, ectopic bone formation, and μ CT analysis

Stem cell implantation-mediated ectopic bone formation was performed as described [25, 26, 29]. Briefly, the iSCAP cells were infected with AdBMP9 or AdGFP, and treated with TNF- α (10ng/ml) condition for 36h, collected and resuspended in PBS with TNF- α (10ng/ml) for subcutaneous injection (5 $\times$ 10⁵ cells/injection) into the flanks of athymic nude (nu/nu) mice (5 animals per group, 4-6 week old, female, acquired from the Laboratory Animal Center, Chongqing, China). At 4 weeks post implantation, animals were sacrificed, and the implantation sites were retrieved for μ CT imaging, histologic evaluation and special stains.

Histological evaluation and Masson's Trichrome staining

The retrieved tissues were fixed, decalcified in 17% EDTA solution, and paraffin embedded. Serial sections of the embedded specimens were deparaffinized and stained with hematoxylin & eosin (H & E). Trichrome staining was also carried out as previously described [25, 29].

Statistical analysis

The quantitative assays were performed in triplicate and/or repeated three times. Data were expressed as mean $\pm$ SD. Statistical significances were determined by one-way analysis of variance and the student's t test. A value of $p < 0.05$ was considered statistically significant.

Results

TNF- α is released during the development of periapical periodontitis and is positively correlated with the area of periapical bone defects

In the process of establishing the animal model of periapical periodontitis of immature permanent teeth with periapical lesions, we found that the periapical alveolar bone loss after 3-days pulp exposed (Fig. 1A). Lesion area was gradually increased from day 3 to day 14, and then sharply increased until 28 days post exposure. The lesion area kept rather stably from day 28 to day 35. The bone loss area of mesial root apical alveolar was also calculated. Quantitatively, the rapid periapical bone absorption was from day 14 to day 28 ($p < 0.01$) (Fig. 1B).

Histological evaluation showed that the apical foramens were not closed and large quantities of inflammatory cells were observed from day 7 to day 28. The bone boss area was filled with granular tissue, and the trend of lesions area development was consistent with that observed by imaging analysis (Fig. 2A). The immunohistochemical analysis indicated the expression of TNF- α was gradually increased from day 3 to day 7. The expression of TNF- α was significantly elevated from day 14 to day 28 and descended from day 28 to day 35. Thus, the expression of TNF- α was seemingly correlated well with the area of periapical bone defects. These results suggest that TNF- α may be primarily released during the development of periapical lesions and positive correlated with the area of periapical bone defects (Fig. 2B).

TNF- α inhibits BMP9-induced osteoblastic differentiation of iSCAPs

We found that the addition of TNF- α suppressed the ALP activity in BMP9-induced iSCAPs on day 3 and day 5 ($p < 0.01$) (Fig. 3A-B). Furthermore, higher concentrations of TNF- α (10ng/ml and 100ng/ml) significantly suppressed the ALP activity, compared to that at the lower concentrations (0.1ng/ml, and 1ng/ml) (Fig. 3A-B), suggesting that the inhibition capability of TNF- α on BMP9-induced ALP activity may be dose-dependent. The qPCR analysis showed that the expression of the late osteoblastic markers OCN and OPN on day 5 was significantly reduced in the presence of TNF- α (10ng/ml, 100ng/ml) ($p < 0.01$) (Fig. 3 C). Furthermore,

Fig. 1. Imaging analysis of the development of the periapical. The rat model of immature permanent teeth with periapical lesions was created to investigate the development of periapical lesions. (A) The images of first molars with pulp exposed on days 0(control), 3, 7, 14, 21, 28, 35. Representative images are shown. (B) The radiolucent area of mesial root apical (bone loss area) was calculated using μ CT. Each sample was done in triplicate. The result was shown on the images. “***”, $p < 0.01$. “#”, $p > 0.05$.

Fig. 2. TNF- α is largely released during the periapical lesions development and is positively correlated with the area of periapical bone defects. (A) Histologic analysis of the periapical tissue on days 0(control), 3, 7, 14, 21, 28, 35. Representative images are shown. (B) Immunohistochemical detection of TNF- α expression in periapical lesions after μ CT analysis on days 0(control), 3, 7, 14, 21, 28, 35. Representative images are shown. TNF- α -positive area is shown dark brown.

Alizarin Red S staining was also carried out to test the late stage mineralization and found that the TNF- α treatment profoundly inhibited *in vitro* mineralization, compared with the control groups (Fig. 3 D). Collectively, these results strongly suggest that TNF- α may inhibit osteoblast differentiation of iSCAPs in a dose-dependent manner.

Fig. 3. Higher titers of AdBMP9 can partially overcome the inhibition of osteogenesis by high concentration of TNF- α

We further investigated if the increase in the titers of AdBMP9 could overcome the inhibitory effect of osteogenic activity by a high concentration of TNF- α (10ng/ml). When SCAPs were transduced with the increasing titers of AdBMP9, we found that there was a trend of increasing ALP activities at both days 3 and 5 ($p < 0.01$) (Fig. 4A-B) although ALP activities were not fully recovered, compared to that of the control group ($p < 0.01$) (Fig. 4A-B). Accordingly, we found the expressions of OCN and OPN were gradually up-regulated with the increasing AdBMP9 titers while at the highest titers the relative expression of OCN and OPN was still lower than that of the control's (Fig. 4 C). Furthermore, Alizarin red S staining results were consistent with the ALP and qPCR analysis findings (Fig. 4 D). Thus, these results suggest that higher levels of BMP9 may partly overcome the inhibitory effect of high concentration of TNF- α on osteogenesis.

Fig. 3. TNF- α inhibits the BMP9-induced osteogenic markers in iSCAPs. (A) ALP histochemical staining assay. Five doses of TNF- α (0 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml) were assayed to iSCAPs infected with AdBMP9 or AdGFP. ALP staining was assayed on day 3 and day 5. Representative images are shown. (B) Quantitative ALP assay. The iSCAPs were seeded in 24-well plates and the treatment conditions were same to that in (A). Each assay condition was done in triplicate. “**”, $p < 0.01$. (C) Relative mRNA expression of OCN (a) and OPN (b) were calculated by qPCR. GAPDH was used as reference gene. “**”, $p < 0.01$. (D) Alizarin Red S staining. The mineralization staining assay

was done on day 21. iSCAPs infected Ad-BMP9 or Ad-GFP treated with five doses of TNF- α . Representative images are shown.

High concentration of TNF- α impairs the BMP9-induced osteogenic differentiation of iSCAPs. Using our previously established stem cell implantation assay [25, 26, 29], we tested the effect of high dose TNF- α (10 ng/ml) on BMP9-induced ectopic bone formation in iSCAPs. Cells were transduced with AdBMP9, or AdGFP, and incubated with or without TNF- α (10 ng/ml) and injected subcutaneously into the flanks of athymic nude mice for 4 weeks. No detectable masses were retrieved from the AdGFP or TNF- α alone group. Robust bone masses were retrieved from BMP9 transduced iSCAPs group, while smaller masses were recovered from the BMP9 plus TNF- α groups as the μ CT imaging results revealed the distinct size differences among these samples (Fig. 5A, panel a). Quantitative analysis of the total bone volumes indicates that BMP9 transduction induced significantly more robust bone formation than that transduced with BMP9 in the presence of TNF- α (10 ng/ml) ($p < 0.01$) (Fig. 5A, panel b), suggesting that TNF- α (10 ng/ml) may inhibit BMP9-induced bone formation. Accordingly, H & E histological evaluation revealed that BMP9-transduced cells formed apparent trabecular bone, which was significantly reduced in the presence of TNF- α (10 ng/ml) (Fig. 5B, panels a vs. b). Trichrome staining confirmed that iSCAPs transduced

Fig. 4. Increased AdBMP9 titers can partially reverse the suppression of osteogenic marker expression by high concentration of TNF- α (10ng/ml) in iSCAPs. (A) ALP histochemical staining assay. AdBMP9 or AdGFP infection was done in the indicated titers (MOIs) with or without high TNF- α (10ng/ml) condition. ALP staining assays were assayed on day 3 and day 5. Representative images are shown. (B) Quantitative ALP assay. The iSCAPs were seeded in 24-well plates and the treatment conditions were the same to that described in (A). Each assay condition was done in triplicate. “***”, $p < 0.01$. (C) Relative mRNA expression of OCN (a) and OPN (b) was determined by qPCR. GAPDH was used as the reference gene. “***”, $p < 0.01$. (D) Alizarin Red S staining. The mineralization staining assay was done on day 14 after the infection of AdBMP9 or AdGFP with or without high TNF- α (10ng/ml) treatment. Representative images are shown.

Fig. 5. TNF- α (10ng/ml) suppresses BMP9-induced ectopic bone formation from iSCAPs *in vivo*. (A) Gross images and μ CT analysis. At 4 weeks after injection, the animals were sacrificed. Masses formed at the injection sites were retrieved, fixed in 4% paraformaldehyde, and subjected to μ CT imaging. No masses were detected the animals injected with AdGFP or treated TNF- α alone group. The 3-D reconstruction was performed for all scanned samples (a), and the average total bone volume was calculated (b) “***”, $p < 0.01$. (B) H & E staining and Trichrome staining of the BMP9 group (a) and the BMP9 plus TNF- α group (b). Representative results are shown. TB, trabecular bone; MBM, mineralized bone matrix; OM, osteoid matrix.

with BMP9 formed more mature and well-mineralized bone matrices, while the maturity and mineralization were significantly diminished in BMP9 with TNF- α group (Fig. 5B, panels a vs. b). Taken together, these *in vivo* results strongly suggest that high dose of TNF- α (e.g., 10ng/ml) may significantly inhibit the BMP9-induced bone formation.

Discussion

During therapeutic bone regeneration, the defective or injured tissues are frequently associated with the presence of abnormal inflammatory and protein mediators [30]. Thus, tissue regeneration may be compromised under chronic inflammation [31]. Wang and others previously demonstrated that BMP9 is one of the most potent of osteogenic differentiation factors in mesenchymal stem cells and in iSCAPs [18, 22, 23, 32, 33]. However, it remains unclear if BMP9-induced bone formation will be affected under inflammatory conditions. Here, we investigated the expression of TNF- α in periapical lesions *in vivo*. Furthermore, we used exogenous TNF- α to simulate the inflammatory microenvironment and evaluate its effects on BMP9-induced osteogenic differentiation of iSCAPs. Our results demonstrate that BMP9-induced osteogenic differentiation can be inhibited by TNF- α in a dose-dependent fashion.

TNF- α is one of the most potent pro-inflammatory cytokines during the inflammation phase. We established an animal model of immature permanent tooth with periapical lesions to observe TNF- α expression during the development of periapical lesions. Our results showed that TNF- α expression was highly correlated with the development of periapical periodontitis. Similarly, it was reported that the expression of TNF- α was positively correlated with periapical damage development during the inflammatory phase in rat models [34]. In addition, our results were also consistent with several clinical studies in which the levels of TNF- α were shown to increase significantly in tooth with periapical pathosis, from smaller to larger lesions [35, 36]. These results suggest that TNF- α may play an active role in periapical bone destruction progress during periodontitis.

It was reported that TNF- α can inhibit osteogenic differentiation and bone formation [9, 37, 38]. It was shown that high concentrations of TNF- α suppressed the expression of osteogenic indicators in MSCs [9, 19, 39]. TNF- α was shown to inhibit osteoblasts differentiation by suppressing the expression of Runx2 in pre-osteoblastic cells [40-43]. However, the effects of TNF- α on osteogenic differentiation of SCAPs remain unclear. Here, we demonstrate that osteogenic differentiation of SCAPs was effectively repressed in the presence of high dose of TNF- α . Thus, TNF- α should be considered as an important negative regulator of bone regeneration under inflammatory microenvironment. As BMP9 has been proven to be one of the most potent osteogenic factors to promote bone defect repair and regeneration [22, 44], it is conceivable that BMP9 may be explored as an agent to combat the inhibitory effect of TNF- α on osteogenic differentiation and induction of bone lesions during chronic inflammation.

Our results suggest that high doses of TNF- α (10ng/ml, 100ng/ml) significantly inhibit BMP9-induced osteogenic differentiation of iSCAPs. Similarly, it was reported that the BMP2-induced osteoblastic differentiation of MSCs was inhibited by TNF- α through activation of TGF- β /Smad signaling pathway [19, 44, 45], while other studies suggest that TNF- α inhibited BMP/Smad signaling through the activation of NF- κ B signaling pathway [46-48]. Nonetheless, the molecular mechanisms underlying such inhibition remain to be thoroughly investigated.

Interestingly, in present study we found that an increase in the titers of AdBMP9 in presence of a constant high concentration of TNF- α (10ng/ml) can partially overcome the inhibitory effect exerted by TNF- α in osteoblastic differentiation of iSCAPs. Similarly, an earlier study reported that TNF- α and BMP2 had opposing effects on osteoblastic differentiation of C2C12 and MC3T3-E1 cells [19]. While the exact mechanisms need to be understood, it offers a promise that exogenous BMP9 may be used to promote the bone defect repair and bone regeneration in the presence of high doses of TNF- α or under chronic inflammation conditions.

In summary, our results strongly suggest that TNF- α may play an important role in periapical bone defect during the inflammatory phase. High doses of TNF- α inhibit the BMP9-induced osteoblastic differentiation of iSCAPs, and the inhibitory effect of TNF- α can be partially reversed by an overexpression of BMP9. Therefore, our results strongly suggest that BMP9 may be further explored as a potent osteogenic factor to improve the efficacy of osteoblastic and odontogenic regeneration in tooth engineering under chronic inflammation conditions.

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Disclosure Statement

The authors declare no competing financial interests.

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