Original Article
Interleukin 1β and tumor necrosis factor α promote hFOB1.19 cell viability via activating AP1

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Abstract: Bone trauma healing is a complex physiological process, which may involve the function of various inflammatory cytokines. Our study aimed to explore the roles of inflammatory cytokines in bone trauma healing and reveal the potential mechanism. Concentrations of interleukin (IL)-6, IL-1β and tumor necrosis factor alpha (TNF-α) in peripheral blood serum of bone trauma patients after surgery were determined by ELISA. The human osteoblast hFOB1.19 cell line was cultured to determine the effect of these cytokines in cell viability using MTT assay. In addition, luciferase reporter assay was performed to investigate the activator protein 1 (AP1) transcriptional activity, and small interfering RNA was transfected to inhibit FOS, a component of AP1 molecule. IL-6, IL-1β and TNF-α exhibited higher level in patients with more severe bone traumas after surgery. IL-1β and TNF-α, but not IL-6, induced a significant increase of hFOB1.19 viability after three days of treatment ($P < 0.05$). IL-1β and TNF-α could activate AP1 transcriptional activity in hFOB1.19 cells ($P < 0.001$), but the activation was inhibited when cells were pretreated with inhibitor of JNKs, SP600125 ($P < 0.001$). Besides, the effect of IL-1β and TNF-α on promoting viability was significantly inhibited after knockdown of FOS. These findings indicated that IL-1β and TNF-α played an important role in promoting osteoblast viability via the activation of AP1 transcriptional activity, which was likely to involve the JNK/MAPK signaling pathway. Modulating inflammatory cytokines is a potential strategy for improving the outcome of bone trauma healing.

Keywords: Bone trauma, cell viability, interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), activator protein 1 (AP1)

Introduction

Bone trauma such as fracture is common, but bone trauma healing is a complex physiological process, which is still an unknown cascade of complex biological events [1]. The initial inflammatory response is considered to be the first step of bone remodeling during bone repair [2], with the stimulated expression of several inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor alpha (TNF-α), and these cytokines subsequently promote angiogenesis and recruit inflammatory cells for bone repair [3, 4]. Thus, an understanding of the inflammatory and repair mechanisms underlying bone trauma healing is informative for the future advancement of bone trauma treating strategies.

Over the past years, a growing body of evidence has indicated that inflammation is an immediate response that play a crucial role in healing after bone trauma [5, 6]. Inflammatory cytokines, such as TNF-α and IL-1α were reported to elevate the expression of the receptor activator of nuclear factor (NF)-κB and osteoprotegerin in human microvascular endothelial cells, and thus play a critical role in human osteoclastogenesis [7]. Several pro-inflammatory cytokines are demonstrated to inhibit osteogenic differentiation from stem cells and have implications for bone repair during inflammation [8]. Besides, Mountziaris et al. discussed the effects of anti-inflammatory agents on bone healing and suggested that harnessing and modulating inflammation might be therapeutic strategies for bone regeneration [9]. It can therefore be speculate that identification of inflammatory signals will help to develop effective agents for bone healing. Although a tremendous amount of efforts have been made to elucidate the relationship between inflammatory cytokines and bone trau-

ma healing, the regulatory mechanisms involved are still largely unknown.

This study aimed at revealing the relationship between inflammatory cytokines and bone trauma healing, as well as the underlying mechanism. We analyzed three inflammatory factors, IL-6, IL-1β and TNF-α, whose functions have been reported related to bone repair processes [10-12]. Their concentration changes in peripheral blood serum samples of bone trauma patients after surgery were detected. Human osteoblast cell line hFOB1.19 was treated with these inflammatory cytokines to determine their effects on cell viability using MTT assay. Besides, phorbol ester PMA, SP-600125 (an inhibitor of c-Jun N-terminal kinases) and small interfering RNA (siRNA) transfection were used to uncover the possible functional mechanism of these cytokines. This study will provide a new insight into the molecular mechanism of bone trauma healing, and may help to develop novel strategies for accelerating the healing process, thus improving clinical outcome.

Materials and methods

Peripheral venous blood samples

A total 26 trauma patients aging 21-78 years (49.0 ± 15.7) were diagnosed with limb-fracture (15 cases), multiple fractures (8 cases) or needing pelvic or joint replacement (3 cases) in our hospital from March 2014 to February 2015. The severity of injury was evaluated preoperatively according to the Injury Severity Score (ISS) [13], and all patients were then categorized into minor (9 cases), moderate (8 cases) and severe (9 cases) groups according to the severity of bone trauma. The position of bone trauma was not statistically significant between groups ($P > 0.05$). All patients underwent surgical treatment, including percutaneous compression plate and dynamic hip screw. The four situations were not found in these patient: phlebothrombosis or coagulation disorders, blood/liver/renal diseases or tumor histories, diabetes, and open bone traumas. Ten healthy cases aging 23-71 years (44.5 ± 15.6) were also sampled during the corresponding period and used as the control group. All the four groups possessed no significant difference in the age or gender. For each case, 4 mL peripheral venous blood was sampled at 0, 1, 3, 5 and 7 days after surgery. This study was approved by the local ethics committee, and written informed consent was obtained from each individual for the use of their blood samples for research.
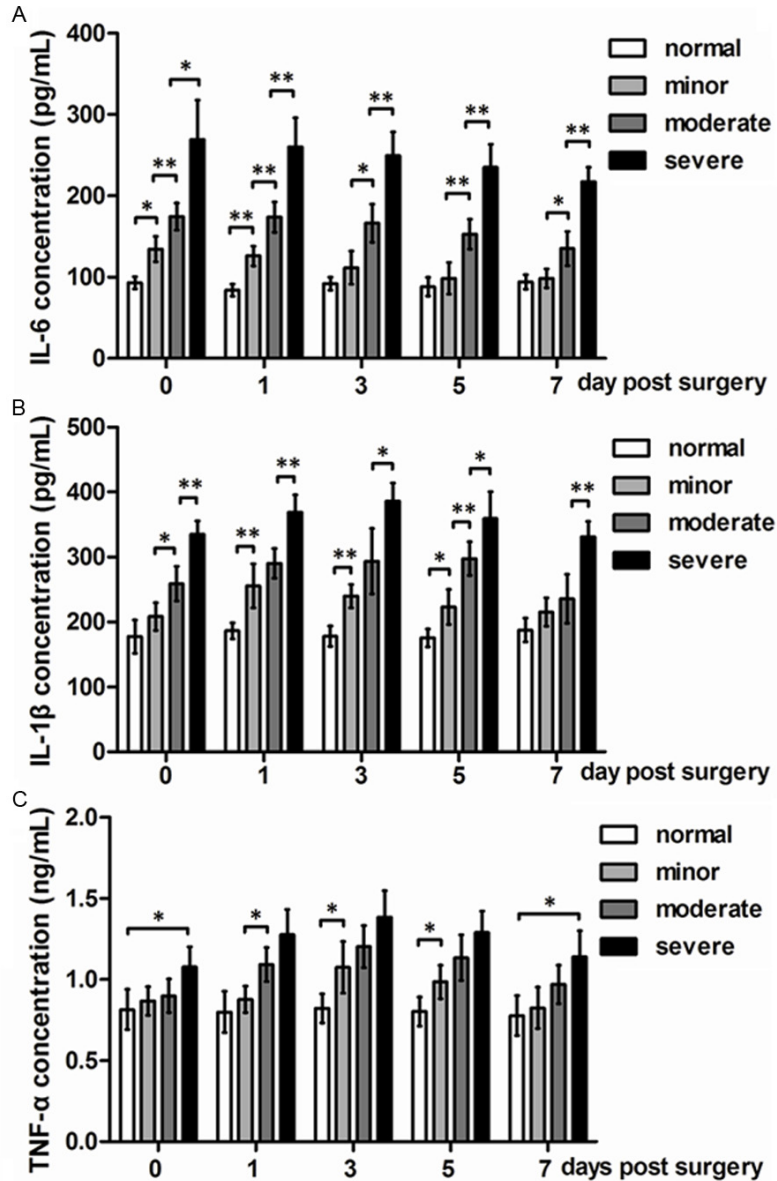
ELISA

Peripheral venous blood samples were centrifuged for 10 minutes at 2,000 g, and serum was transferred into sterile tubes for further analysis. Concentrations of IL-6, IL-1β and TNF-α in serum were determined using a sandwich enzyme-linked immunosorbent assay (ELISA, MultiSciences Biotech, Hangzhou, China) according to the manufacturer's instructions. In brief, samples were first incubated using diluted antibody solution for 1 h. After washing, HRP solution was added to incubate these samples for another 1 h. Afterwards, TMB solution was added to incubate for 15 min at dark. After termination of the reaction with 4N sulfuric acid, the optical density (OD) of the samples at 450 nm was determined using a microplate reader within 5 minutes.

hFOB1.19 cell culture and treatment

The human osteoblastic cell line hFOB1.19 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and G418 (0.3 mg/mL, Gibco, Carlsbad, CA, USA) and maintained in a humidified atmosphere with 5% CO_2 at 37°C. The medium was replaced twice a week and the cells were passaged at confluence of 80%. Cells were treated with IL-1β (1 ng/mL, PeproTech, Rocky Hill, NJ, USA), IL-6 (1 ng/mL, PeproTech), TNF-α (1 ng/mL, PeproTech), PMA (10 ng/mL, Sigma-Aldrich, Shanghai, China) and SP600125 (10 μM, Sigma-Aldrich).

Cell viability assay

Cell viability was measured by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) using Cell Proliferation Kit I (Roche, Basel Switzerland). In brief, hFOB1.19 cells (5 × 10^4 cells/ml) at the logarithmic phase from different groups were seeded in 96-well plates for 10 h, and treated with IL-1β, IL-6 or TNF-α, and the assay was conducted at 0, 1, 2, 3 and 5 days after treatment. Subsequently, MTT was added into each well with a final concentration of 100 μg/mL for 4 h. Acidic isopropanol was

Figure 1. ELISA analysis shows the concentration change of IL-6, IL-1β and TNF-α in peripheral blood serum samples of bone trauma patients after surgery. Samples were taken at 0, 1, 3, 5 and 7 days after surgery. *$P < 0.05$. **$P < 0.01$. Normal, samples of healthy individuals. Minor, samples of patients with minor bone traumas. Moderate, samples of patients with moderate bone traumas. Severe, samples of patients with severe bone traumas. IL-6, interleukin 6. IL-1β, interleukin 1 beta. TNF-α, tumor necrosis factor alpha. A. Concentration of IL-6 in peripheral blood serum. B. Concentration of IL-1β in peripheral blood serum. C. Concentration of TNF-α in peripheral blood serum.

ding to the manufacturer's instructions. In brief, cells were seeded in 24-well plates (3×10^4 cells per well) coated with poly-D-lysine (BD Biosciences, San Jose, CA, USA). Then cells were transfected with AP1 reporter, negative control and positive control. Twenty-four hours later, medium was replaced by assay medium (Opti-MEM + 0.5% FBS + 0.1 mM NEAA + 1 mM Sodium pyruvate + 100 U/ml penicillin + 100 μg/ml streptomycin). Cells of five SP600125-treated groups were pre-treated with SP-600125 for 10 h, and then were treated with IL-1β, IL-6, TNF-α or PMA for 18 h according to the experimental design. Dual Luciferase assay was performed 48 h after transfection to measure pathway signaling activity using GloMax (Promega, Madison, WI, USA). A Renilla reporter was used for internal normalization.
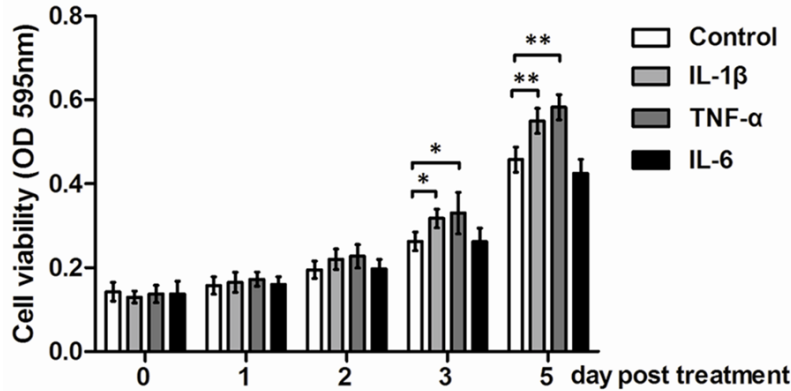
siRNA transfection

Inhibition of FBJ murine osteosarcoma viral oncogene homolog (*FOS*) expression was performed by the transfection of its specific siRNA (Ambion, Huntingdon, UK). The control and FOS-specific siRNAs were transfected into the cells with 5 d of IL-1β or TNF-α treatment using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Then MTT assay was used to measure cell viability as described above.

Statistics analysis

All experiments were performed in triplicate, and the data were presented as the mean ± standard deviaton and the statistical analyses

then added to stop the reaction and OD at 595 nm was measured with an ELISA reader (Bio-Rad, Hercules, CA, USA).

Luciferase reporter assay

AP1 transcriptional activity in the treated cells was measured using Cignal AP1 Reporter (luc) Kit (CCS-011L, Qiagen, Shenzhen, China) accor-

Figure 2. MTT assay shows the effects of IL-6, IL-1β and TNF-α on hFOB1.19 cell viability during an experimental period of 5 days. No significant change is observed in the first two days. Up-regulation of cell viability is observed after three day of treatment with IL-1β or TNF-α, but not IL-6. *$P < 0.05$. **$P < 0.01$. Control, cells with no treatment. IL-6, interleukin 6. IL-1β, interleukin 1 beta. TNF-α, tumor necrosis factor alpha.
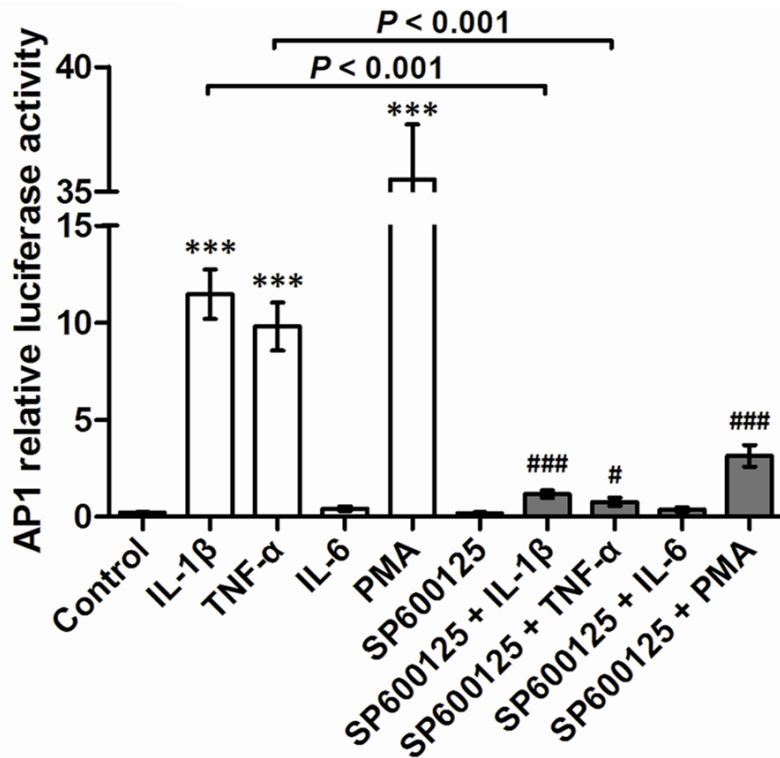
Figure 3. Relative luciferase activity of AP1 in cells treated with IL-1β, TNF-α, IL-6, PMA or SP600125. When cells are pretreated with SP600125, the effect of IL-1β and TNF-α is inhibited ($P < 0.001$). ***$P < 0.001$ compared to the Control group (cells with no treatment). #$P < 0.05$ compared to the PMA group (cells treated with PMA). ###$P < 0.001$ compared to the PMA group. AP1, activator protein 1. IL-6, interleukin 6. IL-1β, interleukin 1 beta. TNF-α, tumor necrosis factor alpha. PMA, the protein kinase C-activating phorbol ester, used as the positive control. SP600125, a selective inhibitor of JNKs.

were performed using SPSS20 software. The differences between groups were compared

using *t*-test or one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

The concentration change of inflammatory cytokines during bone trauma healing

The concentration of IL-6, IL-1β and TNF-α in the peripheral blood serum samples of patients was analyzed using ELISA kits. The three factors showed disparate changing patterns during the 7 days after surgery (**Figure 1**). The IL-6 concentration in patients was gradually decreased (**Figure 1A**), but the IL-1β and TNF-α concentrations possessed a slight increase before they were decreased at 5 d after surgery (**Figure 1B** and **1C**). In addition, the IL-6 concentration in healthy individuals was about 100 pg/mL, but it was much higher in patients, increasing with the trauma severity ($P < 0.05$ or $P < 0.01$). Similarly, patients with more severe trauma also had higher IL-1β and TNF-α concentration. These results suggested that the three inflammatory cytokines might play vital roles in the regulation of cellular activities during bone trauma healing, which should be further studied.

IL-1β and TNF-α promote hFOB1.19 cell viability

We next investigated the roles of the three factors by detecting the human osteoblast cell hFOB1.19 cell viability after IL-6, IL-1β or TNF-α treatment. Significant promotion of cell viability was found
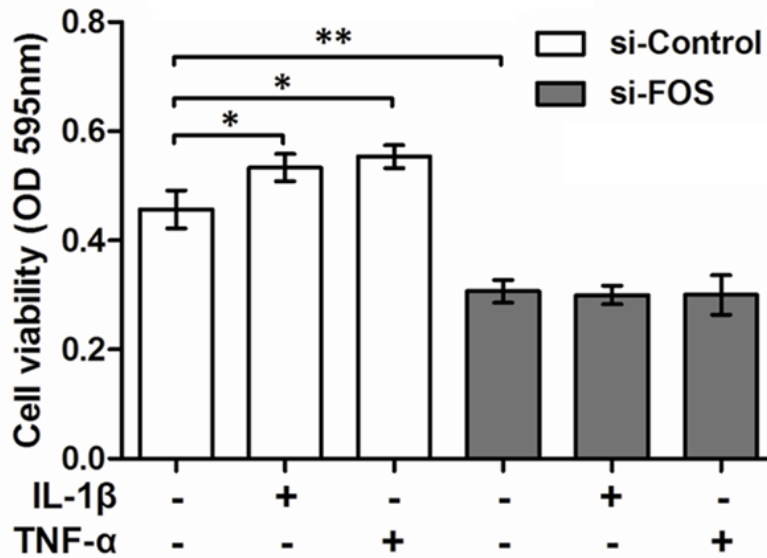
Figure 4. AP1 is necessary for the function of IL-1β and TNF-α in promoting hFOB1.19 cell viability. Cells are transfected with FOS-specific siRNA (si-FOS) or the control (si-Control). When FOS is inhibited, IL-1β or TNF-α treatment cannot promote cell viability. *$P < 0.05$. **$P < 0.01$. IL-1β, interleukin 1 beta. TNF-α, tumor necrosis factor alpha. FOS, FBJ murine osteosarcoma viral oncogene homolog.

activity as predicted ($P < 0.001$). Moreover, IL-6, IL-1β and TNF-α treatment could also activate the AP1 transcriptional activity when cells were pretreated with SP600125 ($P < 0.001$), which inhibits c-Jun N-terminal kinases (JNKs) of mitogen-activated protein kinase (MAPK) pathways, but the effect was not as significant as only using IL-1β or TNF-α, suggesting that the JNK/MAPK pathway might be involved in the IL-1β and TNF-α-induced activation of AP1. Taken together, these results indicated IL-1β and TNF-α might activate AP1 in hFOB1.19 cells, possibly via the JNK/MAPK pathway, while IL-6 could not activate AP1.

after three-day-treatment of IL-1β or TNF-α ($P < 0.05$), and the effects were more obvious after five days of treatment compared to the untreated cells ($P < 0.01$, **Figure 2**). But the viability of IL-6-treated cells almost remained unchanged. Besides, no disparity was found between the effects of IL-1β and TNF-α. These results indicated IL-1β and TNF-α might help to promote osteoblast cell viability, and the effects was obvious after treatment of three days. However, IL-6 did not show the pro-viability effect.

IL-1β and TNF-α activate AP1

Existed studies have indicated that IL-1 and TNF-α are involved in the activation of AP1 [14, 15], so we started the mechanism study of IL-1β and TNF-α by analyzing the transcriptional activity of AP1 induced by these inflammatory factors. As shown in **Figure 3**, IL-1β and TNF-α treatment significantly increased the relative luciferase activity of AP1 compared with control ($P < 0.001$) while IL-6 treatment could not, indicating that IL-1β and TNF-α, but not IL-6, could activate the AP1 transcriptional activity in hFOB1.19 cells. PMA, a protein kinase C-activating phorbol ester which can activate AP1 and NF-κB, was used as the positive control, and generated obvious up-regulation of AP1

IL-1β and TNF-α promote hFOB1.19 cell viability via AP1

To determine whether AP1 was necessary for the roles of IL-1β and TNF-α in promoting hFOB1.19 cell viability, we performed down-regulation of *FOS* gene using its specific siRNA to inhibit AP1, since the heterodimer composed of FOS and JUN is the dominant form of AP1 in mammals. Results showed that the effect of IL-1β and TNF-α on promoting hFOB1.19 cell viability was significantly inhibited after knock-down of FOS (**Figure 4**), while the cell viability of si-Control was promoted by IL-1β or TNF-α, as the abovementioned results (**Figure 2**), suggesting the necessity of integrate AP1 molecule for the pro-viability function of IL-1β and TNF-α in hFOB1.19 cells.

Discussion

Various studies have demonstrated that the local and systemic concentrations of certain cytokines are increased during fracture healing [3, 16]. In the present study, we explore the potential roles of inflammatory cytokines in human osteoblast hFOB1.19, as well as the possible regulatory mechanism. IL-6, IL-1β and TNF-α are at higher levels in peripheral blood

serum of patients with more severe bone traumas. IL-1β and TNF-α, but not IL-6, can promote the viability of hFOB1.19 cells via activation of AP1 transcriptional activity, which is likely to be associated with the JNK/MAPK pathway.

The concentration of IL-6, IL-1β and TNF-α in peripheral blood serum were all higher in patients with bone trauma than healthy individuals, but the difference was found in their changing patterns, which might be a hint for the disparity functional mechanism of the three cytokines during bone trauma healing. Specifically, IL-6 possessed a gradually decreasing level after surgery, while IL-1β and TNF-α showed a slight up-regulation before the decrease. Together with the subsequent findings in hFOB1.19 cells indicating that IL-6 possessed no significant effect on cell viability, it could be deduced that the distinct changing patter of IL-6 might suggest its irrelevance with the regulation of cell viability in hFOB1.19. Though the relationship between IL-6 and osteoblast viability was uncertain, it was ascertained that IL-β and TNF-α treatment could promote hFOB1.19 cell viability. Consistent with this, the concentration of IL-β and TNF-α in peripheral blood serum of bone trauma patients was higher after surgery, increasing with the trauma severity, which implied that the up-regulated levels were associated with their pro-viability function in osteoblasts.

In our study, MTT method was used to assess the effects of inflammatory molecules on cell viability, and IL-1β and TNF-α were shown to promote hFOB1.19 cell viability, which were in line with previous findings that key inflammatory molecules including IL-1β and TNF-α, could modulate the proliferation of skeletal cells [17, 18]. IL-1β is shown to be active during the fracture repair process in vivo [18] and can promote osteoblast proliferation during acute bone repair [19]. In addition, TNF-α is shown to mediate bone resorption and to play crucial roles in intramembranous and endochondral bone repair [20, 21]. Also, TNF-α is demonstrated to have the ability to bind to its receptors, TNFr1 and TNFr2, in fracture tissues, and activation of one of these receptors subsequently leads to tissue regeneration and remodeling [22, 23]. Therefore, we speculated that the role of IL-1β and TNF-α in promoting hFOB1.19 cell viability might contribute to bone trauma heal-

ing. We also noticed that in the first three days after IL-1β or TNF-α treatment, the cell viability did not show significant changes, which might infer that the two cytokines were not the direct inducers of cell viability, and that they could act through the regulation of some other factors to influence hFOB1.19 cell viability. So we were inspired to investigate the downstream factors of IL-1β or TNF-α.

PMA is a stimulator of AP1 and NF-κB, and was used as a positive control in testing AP1 transcriptional activity. Results showed it did successfully up-regulated AP1 activity, and IL-1β or TNF-α treatment possessed similar effects. In addition, the effect of IL-1β and TNF-α on promoting hFOB1.19 viability was significantly inhibited after knockdown of FOS, a member of AP1 molecule, confirming that the role of IL-1β and TNF-α in regulating hFOB1.19 cell viability was via the activation of AP1 transcriptional activity. AP1 is demonstrated to convert extracellular signals to regulate specific target genes in bone and immune cells, which is of great importance for its mediation in cell proliferation, differentiation, and apoptosis of osteoblasts and osteoclasts [24, 25]. Jochum et al. also confirmed that AP1 (FOS/JUN), the primary form of AP1 in mammals, was involved in osteoblast differentiation and played a key role in bone formation [24]. Furthermore, TNF-α influencing bone regeneration via the activation of the Fra/AP1 pathway has been studied [26, 27]. In this case, the result in this study was an evidence for the involvement of FOS and AP1 in the modulation of hFOB1.19 cell viability, which was a possible pathway of IL-1β and TNF-α in regulating osteoblast viability.

Further, when JNKs were inhibited by SP60-0215, all the promotive effects were not so significant, indicating the JNK/MAPK pathway was a possible mediator for the two cytokines activating AP1. Previous studies have confirmed that TNF-α can indirectly up-regulate FOS/AP1 via the JNK/MAPK pathway [27]. IL-1β and TNF-α can synergize with the receptor activator of NF-κB (RANK) and subsequently promote osteoclastogenesis. AP1 transcription factors are also shown to be involved in RANK-regulated osteoclastogenesis under the control of JNKs [28], which is associated with osteoblast proliferation and differentiation [29, 30]. Therefore, our results were consistent with previous find-

ings and suggested that the JNK/MAPK signaling pathway might be a potential mechanism involved in osteoblast viability. Moreover, IL-1β and TNF-α were likely to induce the activation of AP1 transcriptional activity via the JNK/MAPK signaling pathway.

Although the pro-viability effect of IL-1β and TNF-α in hFOB1.19 cells were detected in this study, some aspects still needed further investigation. More detailed studies referring to the dose-dependent effects of the two cytokines would be necessary for uncover the thorough mechanisms. It would be of great value to verify the effects of IL-1β and TNF-α in more osteoblast cell lines, and associate the molecular study with the clinical observation of bone trauma healing processes.

In conclusion, our findings indicate that IL-1β and TNF-α may play an important role in osteoblast viability via the activation of AP1 transcriptional activity involving the JNK/MAPK signaling pathway. Modulating inflammatory factors such as IL-1β and TNF-α may be a potential strategy to improve the clinical outcome of bone trauma healing.

Disclosure of conflict of interest

None.

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