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Tumor Necrosis Factor-α Promotes Osteogenesis of Human Bone Marrow-derived Mesenchymal Stem Cells through JNK-dependent Pathway

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Tumor necrosis factor-α (TNF-α) has been implicated in skeletal diseases by promoting bone loss in inflammatory bone diseases. In the present study, we examined the effects of TNF-α on osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs). TNF-α dose-dependently promoted matrix mineralization of hBMSCs with a maximal stimulation at 2 ng/ml. TNF-α increased expression of alkaline phosphatase, which plays a crucial role for the matrix deposition. The TNF-α-stimulated osteoblastic differentiation was not affected by NFκB inhibitors, BAY and SN50. However, a JNK-specific inhibitor, SP600125 completely abolished the TNF-α-stimulated matrix mineralization and expression of alkaline phosphatase. These results suggest that TNF-α enhances osteoblastic differentiation of hBMSCs through JNK-dependent pathway.

Key words – Mesenchymal stem cells, TNF-alpha, differentiation, JNK, bone

Abbreviations used

TNF-α, tumor necrosis factor-α; NFκB, nuclear factor kappa B; JNK, c-Jun N-terminal kinase; hBMSCs, human bone marrow-derived mesenchymal stem cells; RT-PCR, reverse transcription-polymerase chain reaction; ALP, alkaline phosphatase; GST, glutathione-S-transferease;

Introduction

Bone remodeling is controlled by precise machinery that includes functional coupling between osteoblasts and osteoclasts[10,27]. Osteoblasts are bone forming cells originated from bone marrow stromal cells, whereas osteoclasts are bone resorbing cells derived from monocytes and macrophages. Tumor necrosis factor-α (TNF-α) is a multi-functional cytokine secreted primarily by immune cells, such as macrophages, monocytes, neutrophils, and T-lymphocytes[28,29]. TNF-α has been reported to act on skeleton as a double-edged sword by stimulating bone loss and inhibiting bone formation. For instance, TNF-α leads to bone erosion in various inflammatory diseases through stimulating differentiation of monocytes/macrophages to form osteoclasts[8,13,19]. Whereas, TNF-α inhibits bone-forming properties of various osteoblastic cells and suppress expression of osteoblastic markers in a variety of cell types, including MC3T3-E1, ROS.17/2.8, mouse mesenchymal stromal cells, primary osteoblasts[1,12,15,17,26]. In spite of the previous reports suggesting the inhibitory role of TNF-α on bone formation, the action of TNF-α on osteoblastic differentiation of human bone marrow stromal cells has not been less understood.

TNF-α-induced cellular responses can be elicited by TNF-α receptor 1[28,29]. The activated receptor associates with cytosolic proteins that couple to downstream signals. One of the first associations is with the mediator TNF-α receptor-associated death domain, a cytosolic protein that directs the flow of information along two well-known pathways: The first of these activates TNF-α receptor-associated factors 1 and 2, which regulate a large cytosolic complex containing the I kappa B kinases (α and β), IκB and, ultimately, the activation of the nuclear factor kappa B (NFκB) transcription factor[30]. In addition to NFκB activation, TRAFs signal via c-Jun N-terminal kinase (JNK), p38 kinase, and protein kinase C. However, it is still unclear by which TNF-α regulates osteogenic differentiation of pro-osteoblastic cells.

Human bone marrow-derived mesenchymal stem cells (hBMSCs) possess self-renewal capacity, long-term viability, and differentiation potential toward the diverse cell types, such as adipogenic, osteogenic, chondrogenic, and myogenic lineages[2,3,5,22-24]. Therefore, hBMSCs are useful tools for investigation of the signaling pathways in-

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volved in the TNF-α-induced osteoblastic differentiation. In the present study, we show evidences that TNF-α stimulates osteoblastic differentiation of hBMSCs though JNK-dependent pathways.

Materials and Methods

Materials

α-minimum essential medium, phosphate-buffered sali ne, trypsin, and fetal bovine serum were purchased from Invitrogen. Human recombinant TNF-α was from R&D Systems (Minneapolis, MN). SP600125, BAY, and SN50 were from Calbiochem (La Jolla, CA). The anti-phospho-JNK and anti-phospho-c-Jun (Ser63) antibodies were from Cell Signaling Technology (Beverly, MA). Peroxidase-labeled secondary antibodies and the enhanced chemiluminescence kit were purchased from Amersham Biosciences. All other reagents were purchased from Sigma-Aldrich.

Cell culture

Human bone marrow-derived mesenchymal stem cells (hBMSCs) were obtained from individuals undergoing total hip arthroplasty with the patient's consent, as previously described[16]. Mononuclear cells from bone marrow were separated by centrifugation in a Ficoll-Hypaque gradient (density = 1.077 g/cm^3; Sigma), suspended in α-minimum essential medium containing 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin and seeded at a concentration of 1×10^6 cell/cm^2. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. One-week later, when the monolayer of adherent cells has reached confluence, cells were trypsinized (0.25% trypsin; Sigma), resuspended in α-minimum essential medium containing 10% FBS, and subcultured at a concentration of 2,000 cells/cm^2. The monolayer of adherent cells thus evolved was ex vivo expanded by successive subcultivations and used for the experiments described.

Induction of osteogenic differentiation

Osteogenic differentiation was induced by exposure of hBMSCs to osteogenic differentiation medium (10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid in α-minimum essential medium) over 2 weeks, and the extracellular matrix calcification was visualized by Alizarin Red S staining. Briefly, the cells were washed twice with phosphate-buffered saline and fixed with 4% paraformaldehyde for 30 min. The fixed cells were incubated with 2% Alizarin Red S for 10 min with shaking. To minimize nonspecific staining, the cells were rinsed five times with deionized water and once with phosphate-buffered saline for 20 min. The phase contrast images were photographed by a digital camera equipped in an inverted microscope (Leica DM IRB).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Cells were treated as indicated, and total cellular RNA was extracted by the Tryzol method (Invitrogen). For RT-PCR analysis, aliquots of 2 μg of RNA were subjected to cDNA synthesis with 400 U of M-MLV reverse transcriptase (Invitrogen) and 0.5 μg random primers (Promega, Madison, WI). The cDNA, present in 2 μl of this reaction mixture, was amplified with 1.25 U of GoTaq DNA polymerase (Promega, Madison, WI) and 10 pmol each sense and antisense primers as follows: GAPDH 5'-TCCATGACAACTTTGGTATCG-3', 5'-TGTAGCCAAAT TCGTTGTCA-3, alkaline phosphatase (ALP) 5'-TGGAGCT TCAGAAGCTCAACACCA-3', 5'-ATCTCGTTGTCTGAGTA CCAGTCC-3', The thermal cycle profile was as follows: denaturation for 15 s at 95°C, annealing for 30 s at 58-62°C depending on the primers used, and extension for 30 s at 72°C. For semiquantitative assessment of expression levels, each PCR reaction was realized for different cycles ranging from 30 cycles. PCR products were size fractionated on 1.2% ethidium bromide/agarose gel and quantified under UV transillumination. In all figures, only the results obtained with a number of cycles within the range of log amplification are shown.

Real-time RT-PCR

Quantitative PCR was performed and analyzed on a capillary real-time thermocycler (Light-Cycler, Roche Diagnostics, Mannheim, Germany). Amplification was done in the presence of SYBR Green I as follows: in 20 μl of final volume, cDNA was mixed with LightCycler-Fastart DNA Master SYBR Green (Roche Diagnostics), 10 pmol each of forward primer and reverse primer, and 4 mM $MgCl_2$. Glass capillaries were placed into the Light-Cycler rotor, and the following run protocol was used: a pre-denaturing step at 95°C for 10 min, an amplification and

quantification program repeated for 50 cycles at 95°C for 10 s, annealing for 10 s at 52-58°C, depending on the primers used, and extension for 30 s at 72°C. At the end of elongation at each cycle, SYBR Green I fluorescence was measured. Data analysis was performed essentially as indicated by Roche using "Fit Point Method" in the LightCycler software 3.3 (Roche Diagnostics). Relative quantification was made against serial dilution of GAPDH cDNA used as a housekeeping gene.

Western blotting

Confluent serum-starved cells were treated under the appropriate conditions as specified, washed with ice-cold PBS, and then lysed in lysis buffer [20 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 10 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM Na_3VO_4, 30 mM sodium pyrophosphate, 25 mM β-glycerol phosphate, and 1% Triton X-100]. Lysates were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with 5% nonfat milk, the membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated rabbit IgG antibodies by using the enhanced chemiluminescence Western blotting system.

JNK activity assay

JNK activity was measured by the glutathione based pull-down method by using a kit from Cell Signaling Technology (Beverly, MA) according to the manufacturer's directions. Briefly, cell extracts (0.2 mg of protein) were i ncubated with 1 μg of immobilized glutathione-S-transferase (GST)-c-Jun (1-79) fusion protein, centrifuged, washed, and incubated in kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, and 10 μM ATP) for 30 min at 30°C. Phosphorylated substrate was detected by SDS-polyacrylamide gel electrophoresis and immunoblotting by usingthe phospho-specific c-Jun (Ser63) antibody (dilution 1:1000).

Results

TNF-α enhances osteogenic differentiation of hBMSCss

To explore the effect of TNF-α on osteogenic differentiation of hBMSCs, cells were exposed to osteogenic medium containing the indicated concentration of TNF-α. As shown in Fig. 1A, TNF-α dose-dependently increased the mineral density with a maximal increase at 2 ng/ml concentration, as demonstrated by Alzarin Red-S staining. Exposure of hBMSCs to osteogenic medium induced the mineral deposition, and treatment of the cells with TNF-α during osteogenic differentiation drastically increased the formation of mineralized nodules (Fig. 1B). However, treatment of hBMSCs with growth medium containing 10 ng/ml TNF-α did not induce extracellular mineralization of the cells. These results suggest that TNF-α plays a pivotal role in the regulation of osteogenic differentiation.

To explore time dependence of TNF-α-stimulated osteogenic differentiation in hBMSCs, we determined extracellular mineralization in hBMSCs treated with osteogenic differentiation medium in the absence or presence of TNF-α. Incubation of hBMSCs in osteogenic differentiation medium time-dependently increased the mineralization and maximal differentiation occurred on day 25. However, the mineralization was accelerated by continuous treatment of the cells with 10 ng/ml TNF-α during osteogenic differentiation, and maximal mineralization occurred on day 15 (Fig. 1C).

To assess whether continuous treatment of hBMSCs with TNF-α is essential for the differentiation, the cells were pulse-treated with 10 ng/ml TNF-α for 15 days during the indicated time course of in vitro osteogenesis. Pulse treatment of hBMSCs with TNF-α during day 0-3 significantly increased the mineral deposition (Fig. 1D). These results suggest that exposure of hBMSCs to TNF-α during early stage of differentiation is essential for the TNF-α-stimulated osteogenic differentiation.

TNF-α increases expression of alkaline phosphatase in hBMSCs

Since ALP activity, an early marker of osteogenic differentiation, plays an important role in both the osteogenic differentiation and eventual mineralization processes, we next determined expression level of ALP by semi-quantitative RT-PCR. As shown in Fig. 2A, mRNA level of ALP was increased in hBMSCs cultured in osteogenic medium and treatment of the cells with osteogenic medium along with TNF-α treatment further increased the ALP expression. To confirm these results, the mRNA level of ALP was determined by real time RT-PCR. As shown in Fig. 2B, the expression level of ALP was elevated by treatment of the cells with osteogenic differentiation medium together with

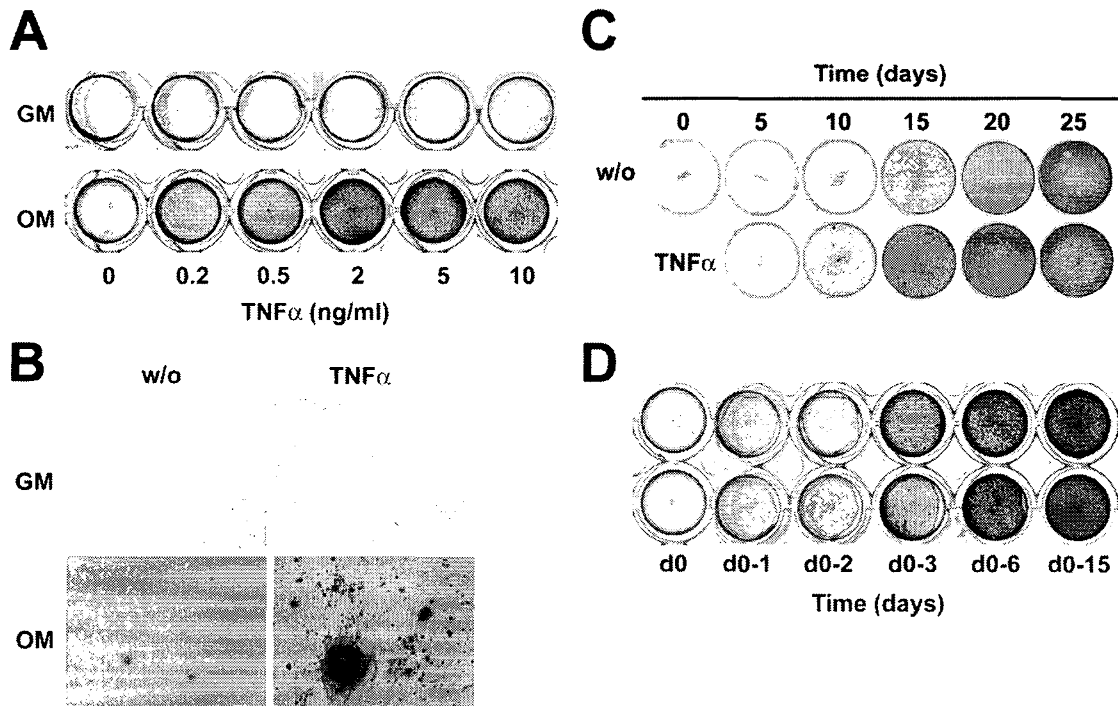

Fig. 1. Effects of TNF-α on Osteogenic Differentiation of hBMSCs. (A) hBMSCs were treated with a growth medium (GM) or osteogenic differentiation medium (OM) in the presence of the indicated concentrations of TNF-α. After 14 days, matrix mineralization was determined by Alizarin Red S staining and photographed by a digital camera. (B) Mineralized nodules in wells were photographed with a digital camera equipped on a light microscopy. (C) hBMSCs were treated with osteogenic differentiation medium in the absence or in the presence of 10 ng/ml TNF-α for the indicated time. Matrix mineralization was determined by Alizarin Red S staining and photographed by a digital camera. (D) During osteogenic differentiation, hBMSCs were pulse- treated with OM containing 10 ng/ml TNF-α for the indicated time periods. After 15 days, matrix mineralization was assessed by Alizarin Red S staining and photographed by a digital camera. Duplicate determinations from three independent experiments were shown.

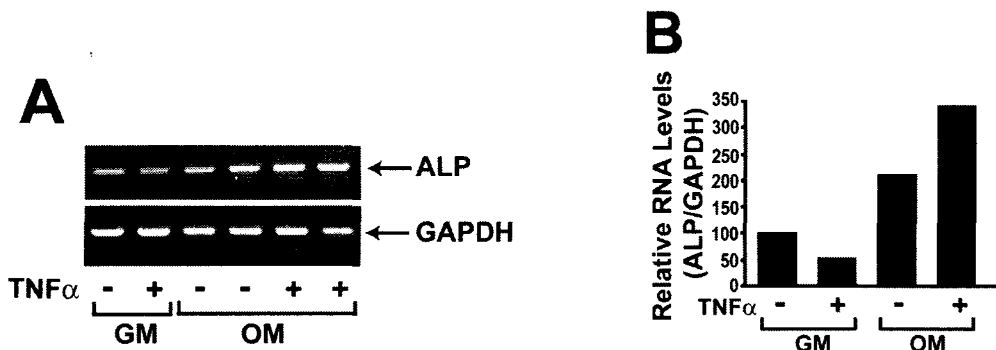

Fig. 2. Effects of TNF-α on the Expression Level of ALP in hBMSCs. (A) hBMSCs were treated with a growth medium (GM) or osteogenic differentiation medium (OM) in the absence or presence of TNF-α for 7 days. The expression levels of ALP and GAPDH were analyzed by RT-PCR. (B) The expression levels of ALP and GAPDH were quantified by qPCR analysis. mRNA levels of ALP were normalized to those of GAPDH. Representative data from three independent experiments were shown.

TNF-α. These results are consistent with the observations that TNF-α treatment stimulated the extracellular mineralization of hBMSCs exposed to osteogenic medium (Fig. 1), suggesting that TNF-α promotes the osteoblastic differentiation by increasing the expression level of ALP.

Molecular mechanism involved in the pro-osteogenic activity of TNF-α

NFκB and JNK are key players of a variety of cellular responses induced by TNF-α[7]. To elucidate the involvement of NFκB in the TNF-α-stimulated osteogensis, we ex-

amined the effects of NFκB inhibitors on the TNF-α-stimulated mineralization. Extracellular mineralization was induced by exposure of hBMSCs to osteogenic medium in the absence or presence of TNF-α and/or NFκB inhibitors. Co-treatment of the cells with NFκB inhibitors, BAY or SN50, had no significant impacts on the pro-osteogenic activity of TNF-α, suggesting that NFκB-dependent pathway is not involved in the TNF-α-stimulated osteogenesis (Fig. 3A). To explore the involvement of JNK in the TNF-α-stimulated osteogenesis, we examined the effect of SP600125, a JNK inhibitor on the TNF-α-stimulated mineralization. As shown in Fig. 3A, pretreatment of the cells with SP600125 completely prevented the TNF-α-stimulated mineralization during osteogenic differentiation. To explore whether JNK plays a role in the TNF-α-stimulated mineralization by enhancing the expression level of ALP, we examined the expression levels of ALP by RT-PCR. As shown in Fig. 3B, SP600125 abrogated the TNF-α-stimulated expression of ALP, suggesting that JNK plays a key role in the TNF-α-stimulated osteogenic differentiation in hBMSCs. Furthermore, TNF-α induced phosphorylation of JNK and c-Jun, and the TNF-α-induced activation of JNK was completely abrogated by pretreatment of the cells with SP600125 (Fig. 3C). These results suggest that JNK plays a crucial role in the increased expression of ALP in response to TNF-α during osteogenic differentiation.

Discussion

TNF-α plays a major role in the progression and severity of bone loss associated with inflammation. TNF-α has been reported to induce bone loss by stimulating bone loss and inhibiting bone formation. For instance, TNF-α leads to bone erosion in various inflammatory diseases through stimulating differentiation of monocytes/macrophages to form osteoclasts[8,13,19]. Whereas, TNF-α inhibits bone-forming properties of various osteoblastic cells and suppress expression of ostoblastic markers in a variety of cell types, including MC3T3-E1, ROS.17/2.8, mouse mesenchymal stromal cells, primary osteoblasts[1,12,15,17,26]. However, in the present study, we demonstrated that TNF-α induces differentiation of hBMSCs into osteoblastic cells, as demonstrated by increased ALP activity

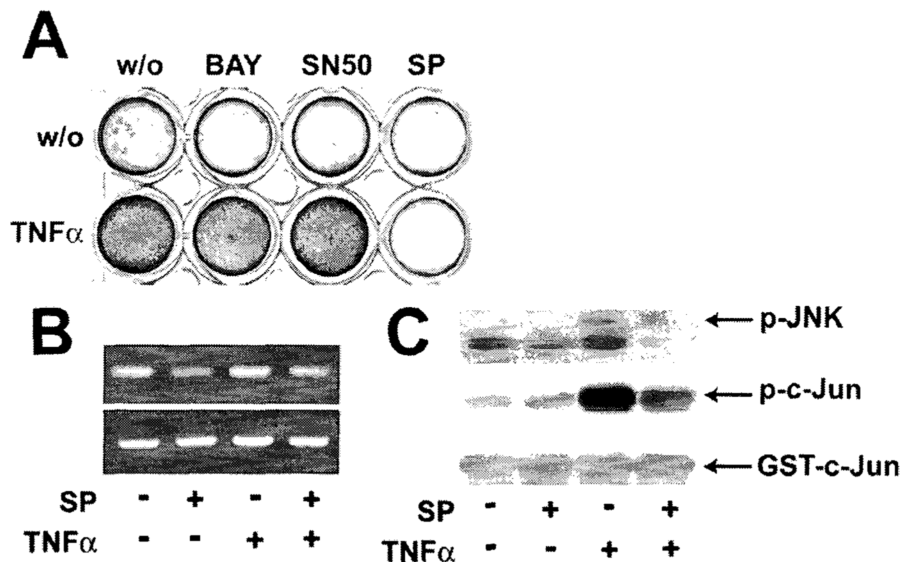

Fig. 3. Role of JNK on the TNF-α-induced Stimulation of Osteogenic Differentiation. (A) hBMSCs were treated with an osteogenic differentiation medium in the absence or presence of 2 μM BAY, 50 ng/ml SN50, 10 μM SP600125, or 10 ng/ml TNF-α as indicated for 14 days. Extracellular mineralization was visualized by Alizarin Red S staining and photographed by a digital camera. (B) The expression levels of ALP and GAPDH were analyzed by RT-PCR. (C) Serum-starved hBMSCs were pre-treated with 10 μM SP600125 for 15 min, and then exposed to 10 ng/ml TNF-α for 10 min. Cell lysates were loaded onto 10% SDS-PAGE and phosphorylation of JNK was determined by Western blotting with anti-phospho-JNK antibody (upper panel). To determine JNK activity, an immobilized GST-c-Jun protein was used to pull down JNK enzymes from cell lysates. Upon addition of kinase buffer and ATP, the phosphorylation of GST-c-Jun was probed by Western blotting by using anti--phospho-c-Jun (Ser63) antibody (middle panel). The amounts of GST-c-Jun were determined by staining with Ponceau S solution (lower panel). Representative data from three independent experiments were shown.

and extracellular mineralization. Therefore, it is likely that TNF-α positively or negatively regulates the osteogenic differentiation, depending on cell types or differentiation stages.

A growing body of evidence suggest that ALP, which is required for normal mineralization, is inhibited by TNF-α by a complex mechanism[4,6,15,20,21,25]. TNF-α regulation of ALP occurs at several levels. Although TNF-α will decrease ALP mRNA and protein, TNF-α can also stimulate ALP release. TNF-α-induced apoptosis of osteoblasts is associated with an immediate release of soluble ALP[9]. TNF-α may also stimulate retinoic acid-mediated increases in ALP gene transcription but simultaneously causes an accumulation of mature mRNA in the cytoplasm, possibly with translational suppression[18]. Furthermore, bone healing after marrow ablation or bone fracture was delayed in the TNF-α receptor ($p55^{(-/-)}/p75^{(-/-)}$) deficient mice. Moreover, type I collagen and osteocalcin mRNA expressions were reduced approximately 30 and approximately 50%, respectively, of the control values in the TNF-α receptor ablated mice[11]. These results support the present study that TNF-α stimulates the ALP-induced matrix mineralization in hBMSCs.

ERK has been reported to play a key role in the regulation of osteoblastic differentiation. For instance, activation of ERK in human osteoblastic cells results in upregulation of expression and DNA binding activity of Cbfa1 or Runx2, the master regulator of osteogenic differentiation [31]. However, implication of JNK in the differentiation of pro-osteoblastic cells to osteoblasts has not been clearly demonstrated. In the present study, we demonstrated that JNK is involved in the TNF-α-stimulated osteogenesis of hBMSCs. Pharmacological ablation of JNK activity completely abrogated the TNF-α-induced pro-osteogenesis in h BMSCs (Fig. 3). Consistently, inhibition of JNK by SP600125 reduced BMP-2-induced production of osteocalcin, which is a marker for osteoblastic differentiation[14]. Taken together, these results suggest a pivotal role of JNK in the TNF-α-stimulated osteoblastic differentiation of hBMSCs.

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초록 : Tumor necrosis factor-α에 의한 골수 유래 중간엽 줄기세포의 골세포로의 분화 촉진에서 JNK의 역할

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Tumor necrosis factor-α (TNF-α)는 염증성 골질환에서의 골조직의 손실과 밀접한 관련이 있다. 본 연구에서는 인체 골수 유래 중간엽 줄기세포의 골세포로의 분화과정에 대한 TNF-α의 영향을 조사하였다. TNF-α는 골수 유래 중간엽 줄기세포의 골세포로의 분화를 나타내는 표시인 세포외 무기질 축적과 alkaline phosphatase의 발현의 증가를 일으켰으며 2 ng/ml의 농도에서 최대의 증가를 나타내었다. TNF-α에 의한 골세포로의 분화는 NFκB의 저해제에 의해서는 영향받지 않았으나 JNK 특이 저해제인 SP600125에 의해 완벽하게 억제되었다. 이는 TNF-α에 의한 골수 유래 중간엽 줄기세포의 골세포로의 분화과정에 JNK가 중요한 역할을 한다는 것을 제시한다.