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FAST TRACK

Activation of c-Jun NH2-Terminal Kinases by Interleukin-1β in Normal Human Osteoblastic and Rat UMR-106 Cells

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Abstract We recently demonstrated the activation of extracellular signal- regulated protein kinase 1 and 2 (ERK1 and ERK2) by IGF-1, FGF-2, and PDGF-BB in normal human osteoblastic (HOB) cells as well as in rat and mouse osteoblastic cells. In this report, we have examined whether c-Jun NH2-Terminal Kinase (JNK) pathway is activated by growth factors and interleukin-1β (IL-1β) in normal HOB and rat UMR-106 cells using immune-complex kinase assay and anti-active JNK antibody, which recognizes activated forms of both JNK1 and JNK2. Results have demonstrated the presence of JNK1 and JNK2 proteins in normal HOB and UMR-106 cells. Both JNK1 and JNK2 were activated by IL-1β. IL-1β preferentially activated JNK pathway in a dose- and time-dependent manner and had little effect on ERK pathway. On the other hand, FGF-2 did not activate JNK but most strongly activated ERK pathway. The activation of JNK was maximal at 20 min whereas maximal activation of ERK1 and ERK2 was observed within 10 min. Results have clearly demonstrated that IL-1β preferentially activates JNK pathway whereas FGF-2 activates ERK pathway in normal human and rat UMR-106 osteoblastic cells. J. Cell. Biochem. 69:87–93, 1998.

Key words: MAP kinase pathways; JNK; human osteoblasts; interleukin-1β; UMR-106 cells

Interleukin-1α (IL-1α) and IL-1β are functionally related cytokines and potent stimulators of bone resorption in vitro and in vivo [Gowen et al., 1983; Stanshenko et al., 1987; Pfeilschifter et al., 1989; Nguyen et al., 1991], and play a pivotal role in the pathogenesis of osteoporosis [Pacifici et al., 1987, 1989; Fujita et al., 1990]. IL-1-stimulated bone resorption requires the simultaneous presence of osteoblasts or osteoblast-conditioned medium, suggesting that osteoblasts are the target cells of IL-1 and mediators of resorptive signal to osteoclasts by releasing cytokines or other unknown soluble factors [Thomson et al., 1986]. In addition, IL-1β has been shown to alter osteoblast expression of bone matrix proteins, type I collagen, osteocalcin and alkaline phosphatase activity [Stanshenko et al., 1987; Hanazawa et al., 1986]. We have demonstrated that IL-1β induces cytokine IL-8 and transcription factor early growth response-1 (Egr-1) gene expression in normal human osteoblastic (HOB) and bone marrow osteoprogenitor stromal cells [Chaudhary and Avioli, 1996; Chaudhary et al., 1996]. The molecular mechanisms involved in gene regulation by IL-1β are poorly understood. Although studies on IL-1 receptor activation have implicated virtually every second messenger pathway, including protein kinase A, protein kinase C, calcium-calmodulin, inositol phosphate hydrolysis, diacylglycerol, eicosanoids, nitric oxide, oxygen radicals [Cunningham and De Souza, 1993], and mitogen-activated protein (MAP) kinases [Guesdon et al., 1993; Ahlers et al., 1994; Wilmer et al., 1997] in different cell types, the exact nature of intracellular signaling pathways that link the activation of the IL-1β receptors, which have no intrinsic protein tyrosine kinase activity, to the biological cellular responses still remains to be defined. Three MAP kinase subfamilies have been identified in mammalian cells: the extracellular signal-regulated kinase (ERK), the c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK), and the p38

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MAP kinase [Cobb and Goldsmith, 1995; Segar and Krebs, 1995; Kyriakis and Avruch, 1996; Su and Karin, 1996]. Environmental stress factors and pro-inflammatory cytokines including IL-1β preferentially activate JNK pathway whereas growth factors activate the ERK pathway [Cobb and Goldsmith, 1995; Segar and Krebs, 1995; Kyriakis and Avruch, 1996; Su and Karin, 1996; Minden et al., 1994; Raingeaud et al., 1995; Mendelson et al., 1996; Scherle et al., 1997]. The activation of ERK pathway by growth factors and PMA has recently been reported in osteoblastic cells [Verheijen and Defize, 1995; Zhang et al., 1995; Siddhanti et al., 1995; Chaudhary and Avioli, 1997a,b]. However, the signalling pathways activated in response to IL-1β as well as JNK pathway have not been described in osteoblastic cells. In this study, we have examined the activation of JNK by IL-1β and FGF-2 in normal human and rat UMR-106 osteoblastic cells.

MATERIALS AND METHODS

Materials

Myelin basic protein (MBP), sodium orthovanadate, 2-mercaptoethanol, Dulbecco's phosphate-buffered saline (PBS), crude bacterial collagenase, trypsin-EDTA, Histopaque-1077, fetal bovine serum (FBS), and Dulbecco's modified Eagle medium (DMEM):Ham's F-12 medium (1:1) were obtained from Sigma (St. Louis, MO). Protein G-Sepharose 4 fast flow was obtained from Pharmacia Biotech Inc. (Piscataway, NJ). Human recombinant platelet-derived growth factor-BB (PDGF-BB) and interleukin-1β (IL-1β) were purchased from R&D Systems (Minneapolis, MN). Polyclonal rabbit antiserum against ERK2 was kindly provided by Dr. John C. Lawrence, Jr. GST-cJun (1-79) was obtained from the Stratagene (La Jolla, CA). Polyclonal rabbit anti-active MAPK antibody, which recognizes activated forms of both ERK1 and ERK2, was purchased from Promega (Madison, WI). Anti-active JNK and anti-JNK antibodies were obtained from New England Biolabs, Inc. (Beverly, MA). FGF-2 was a generous gift from Amgen Corporation (Thousand Oaks, CA).

Human Bone Cell Culture

Human ribs obtained from surgery patients were used to scrape out trabecular bone with a size 4 bone currette and human osteoblast-like (HOB) cells were cultured from trabecular bone chips as described previously [Chaudhary and Avioli, 1996; Chaudhary et al., 1996]. Briefly, bone chips were washed several times with DMEM:Ham's F-12 medium and digested with collagenase (Boehringer Mannheim, Indianapolis, IN; 250 U/ml) and DNase type I (Sigma, 1 µg/ml) in DMEM:F-12 medium for 2 h at 37°C. After digestion, chips were washed with DMEM: F-12 (calcium-free) containing 10% FBS and plated in calcium-free DMEM:F-12 medium containing 10% FBS and penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively) in T-175 culture flasks. Rat UMR-106 cells were cultured in the Eagle's MEM medium. Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Cell Lysate Preparation

Normal HOB and rat UMR-106 cells were trypsinized and seeded in P-100 culture dishes at a density of one million cells/dish. Cells were grown to confluency (48–72 h) and then made quiescent in the medium containing 0.1% BSA for 24 h. The required amounts of concentrated stock solutions of FGF-2 and IL-1β were directly added to the medium for the specified time period. Cells were rinsed with cold PBS containing 100 µM sodium orthovanadate. Cell lysates were prepared by adding 0.5 ml of cold lysis buffer A (50 mM β-glycerophosphate, pH 7.4, 1 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 µg/ml leupeptin, 10 µg/ml aprotinin, 3 mM benzamidine, 1% NP-40, 2 mM EDTA, 1 mM EGTA) to each dish. Cells were incubated for 30 min on ice, cell suspension transferred to microcentifuge tubes, and centrifuged for 15 min at 14,000*g* in a cold room. The supernatants were removed, stored at −80°C and used as the source of enzyme. Proteins in cell lysates were measured with the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA).

Immune-Complex Kinase Assay

Cell lysates (100 µg protein) were immunoprecipitated at 4°C for 1 h with appropriate antibodies and ERK and JNK assayed as described previously [Chaudhary and Avioli, 1997a; Nagata et al., 1997; Read et al., 1997] with slight modifications. This was followed by incubation of the immune-complexes with 30 µl of protein G-Sepharose beads for another 1 h at 4^0C. The beads were then washed three times with cold RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 0.2 mM sodium orthovanadate) and then twice with the cold kinase washing buffer B (25

mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 10 µg/ml aprotinin, 1 mM benzamidine). ERK2 activity was determined by resuspending the immune-complexes in 40 µl of ERK assay buffer C (25 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 20 µM ATP, 500 µg/ml MBP, 10 µg/ml aprotinin, 1 mM benzamidine 0.5 mM EDTA, 2 µCi of [γ-^{32}P]ATP and incubating at 30^{0}C for 30 min. For JNK, beads were washed three times with buffer D (20 mM Tris, pH 7.4, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium vanadate, 2 mM pyrophosphate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 20 mM NaF, and 1 mM PMSF) and twice with buffer E (20 mM HEPES, pH 7.4, 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 0.1 mM sodium orthovanadate, and 1 mM DTT). The beads were resuspended in 40 µl of JNK assay buffer F (20 mM HEPES, pH 7.4, 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 1 mM DTT, 50 µM ATP, 2 µg GST-cJun, and 5 µCi [-^{32}P]ATP and incubated for 20 min at 30^{0}C. Reactions were stopped by adding 20 µl of 3 x SDS sample buffer, samples boiled for 5 min and electrophoresed on 12.5% SDS-polyacrylamide gels. The phosphorylated substrate bands were detected by autoradiography.

Western Blotting (Immunoblotting)

Cell lysates were subjected to SDS-PAGE and proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF)/Immobilin-P membrane (Millipore Corp., Medford, MA) as described [Scherle et al., 1997]. The membranes were incubated in blocking buffer [Tris-buffered saline-0.1% Tween-20 (TBST)] containing 1% BSA for anti-active MAPK at room temperature for 1 h, then incubated with appropriate antibodies according to the protocols of the manufacturers (Promega and Santa Cruz Biotechnology) and washed with TBST. Antibody binding was detected by enhanced chemiluminescence (ECL) Western blotting detection systems as directed by the manufacturer (Amersham, Arlington Heights, IL). Data shown are representative of two individual experiments with similar results.

RESULTS

Time-Course Studies of Activation of JNK and ERK by IL-1β in Normal HOB Cells

To determine whether activation of JNK is time-dependent, quiescent HOB cells were treated with IL-1β (10 ng/ml) for different time periods. Cell lysates were electrophoresed and proteins transferred to Immobilin-P membranes, which were immunoblotted with anti-active JNK antibody. As shown in Figure 1, activated forms of JNK1 and JNK2 were recognized by the antibody which were increased in the presence of IL-1β as compared to non-stimulated control cells. Results showed time-dependent activation of 46-kD JNK1 and 55-kD JNK2 reaching maximal by 20 min and thereafter declining (Fig. 1). These data also supported the importance of anti-active JNK antibody to detect and measure JNK1 and JNK2 activation. The anti-JNK antibody, which recognizes, depending on cell type, both JNK1 and JNK2, serves as an important control to demonstrate the amount of JNK in each lane, thereby allowing accurate interpretation of the corresponding signal obtained with the anti-active JNK antibody. Therefore, the same membrane was stripped and incubated with anti-JNK antibody to determine the amounts of JNK proteins. Anti-JNK antibody recognized 55-kD JNK2 and showed similar JNK2 protein amounts in control and treated samples. It is important to mention that this antibody did not recognize JNK1 in osteoblastic cells since a

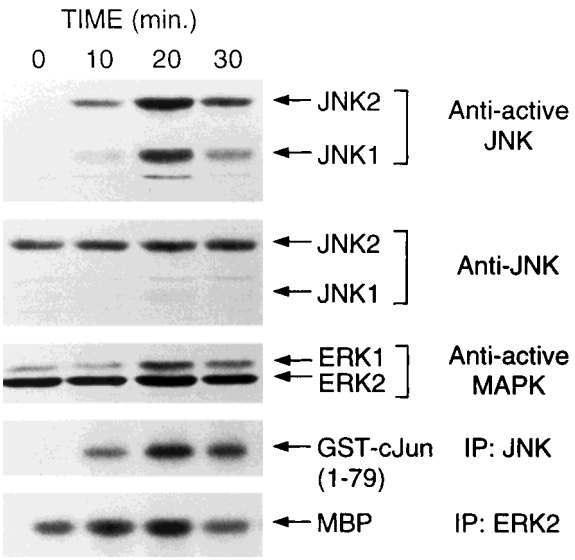

Fig. 1. Time-course studies of JNK and ERK activation by IL-1β in HOB cells. HOB cells were treated with IL-1β (10 ng/ml) for different time periods. JNK and ERK activation was determined by Western blotting with anti-active JNK and anti-active MAPK antibodies and immune-complex kinase assay using [γ-^{32}P]ATP and GST-cJun or MBP as substrates as described in Materials and Methods. The phosphorylated GST-cJun and MBP were detected after SDS-PAGE by autoradiography. Data shown are representative of two individual experiments with similar results.

very faint band of 46-kD JNK1 was observed. It is known that this antibody recognizes either JNK2 or both JNK1 and JNK2 depending on cell type. To further confirm the activation of JNK, the activity was determined by immune-complex kinase assay. The incorporation of [γ-^{32}P]ATP into GST-cJun was increased with time in the presence of immune-complex by IL-1β with maximal activation by 20 min and thereafter declined. IL-1β slightly activated ERK1 and ERK2 as determined by immune-comlex kinase assay and immunoblotting with anti-active MAPK antibody (Fig. 1).

Dose-Response of IL-1β on the Activation of JNK in Normal HOB Cells

Quiescent HOB cells were treated with different doses (0.01, 0.1, 1.0, and 10.0 ng/ml) of IL-1β. Cell lysates were electrophoresed and proteins transferred to Immobilin-P membranes, which were immunoblotted with anti-active JNK antibody. As shown in Figure 2, activated forms of JNK1 and JNK2 were recognized by the antibody that was increased in the presence of IL-1β as compared to non-stimulated control cells. The maximal activation of JNK1 and JNK2 was observed at 1 ng/ml and remained the same at 10 ng/ml without further increase.

Time-Course Studies of JNK Activation by IL-1β in UMR-106 Cells

UMR-106 cells were treated with IL-1β (10 ng/ml) for different time periods. Activation of JNK was determined by immunoblotting with anti-active JNK antibody and immune-complex kinase assay. Both JNK1 and JNK2 were activated in a time-dependent manner reaching maximal by 20 min. The activation of JNK1 was greater than the activation of JNK2 by IL-1β in rat UMR-106 cells (Fig. 3). Similarly, the maximal activation of JNK was also observed at 20 min by immune-complex kinase assay (Fig. 3).

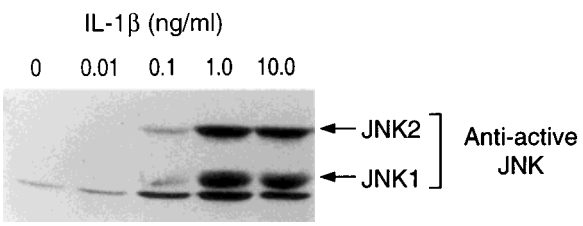

Fig. 2. Dose-response of IL-1β on the activation of JNK. HOB cells were treated with different concentrations of IL-1β for 20 min. Cell lysates were subjected to SDS-PAGE followed by transfer of proteins to Immobilin-P membranes and then incubated with anti-active JNK antibody. The binding of antibody was detected by ECL detection reagents.

Comparison of ERK and JNK Activation by IL-1β and FGF-2 in Normal HOB and Rat UMR-106 Cells

Since ERKs are primarily activated by growth factors and JNKs by environmental stress factors and pro-inflammatory cytokines including IL-1β, experiments were performed to compare the activation of JNK and ERK by FGF-2 and IL-1β. Results showed that IL-1β preferentially activated JNK but had little effect on ERK1 and ERK2. In contrast, growth factor FGF-2 mainly activated ERK1 and ERK2 without affecting JNK activation (Fig. 4). There were differences in the kinetics of activation of JNK by IL-1 β and ERK by FGF-2. The maximal stimulation of JNK was observed at 20 min while ERK at 10 min. Data also showed that anti-active JNK antibody cross-reacted with ERK1 and ERK2 as demonstrated by the detection of two corresponding bands in the presence of FGF-2 in both HOB and UMR-106 cells. Thus, the band observed below the JNK1 band with anti-active JNK antibody in Figures 1, 2, and 4 appears to be the ERK2.

DISCUSSION

In the present study we investigated the activation of MAP kinases by IL-1β in normal human osteoblastic and rat UMR-106 cells. Using Western blotting with anti-active JNK antibody, which recognizes activated forms of JNK1 and JNK2 and an immune-complex ki-

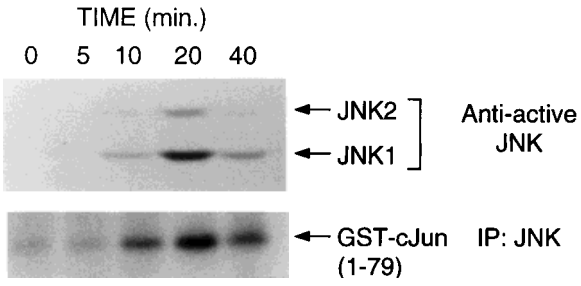

Fig. 3. Time-course studies of JNK activation by IL-1β in UMR-106 cells. UMR-106 cells were treated with IL-1β (10 ng/ml) for different time periods. JNK activation was determined by Western blotting with anti-active JNK antibody and immune-complex kinase assay using [γ-^{32}P]ATP and GST-cJun as substrates. The phosphorylated GST-cJun was detected after SDS-PAGE by autoradiography.

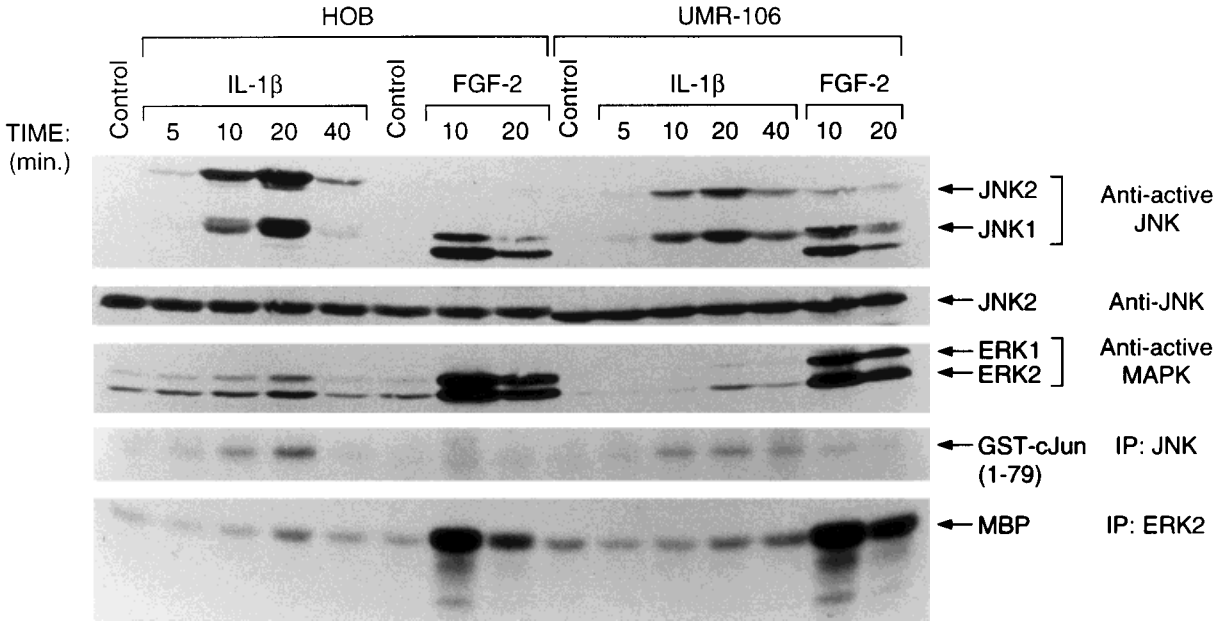

Fig. 4. Comparison of JNK and ERK activation by IL-1β and FGF-2 in HOB and UMR-106 cells. HOB and UMR-106 cells were treated with IL-1β (5 ng/ml) or FGF-2 (50 ng/ml) for different time periods. JNK and ERK activation was determined by Western blotting with anti-active JNK and anti-active MAPK antibodies and immune-complex kinase assay using [γ-^{32}P]ATP, and GST-cJun or MBP as substrates. The phosphorylated GST-cJun and MBP were detected after SDS-PAGE by autoradiography.

nase assay, our results have demonstrated for the first time that IL-1β activates JNK1 and JNK2 in normal HOB and UMR-106 cells. In contrast, an osteogenic growth factor FGF-2 failed to activate JNK1 and JNK2 but most strongly stimulated ERK1 (p44^{MAPK}) and ERK2 (p42^{MAPK}). These results are consistent with the findings that pro-inflammatory cytokines IL-1β and TNF-α preferentially activate JNK [Raingeaud et al., 1995; Read et al., 1997] and growth factors activate the ERK pathway [Cobb and Goldsmith, 1995; Segar and Krebs, 1995; Kyriakis and Avruch, 1996; Su and Karin, 1996]. Our data also showed that FGF-2 activated only ERK1 and ERK2 without activating JNK. Similar results had been reported in the murine fibrosarcoma cell line wherein FGF-2 stimulated only ERK and had no effect on JNK activity [Gardner and Johnson, 1996]. We previously showed that IGF-I, PDGF-BB, and FGF-2 activated ERK1 and ERK2 in human, rat, and mouse osteoblastic cells [Chaudhary and Avioli, 1997a,b]. The accumulated data suggest that the activation of various MAP kinase pathways is important in mediating the biological responses to IL-1β cytokine and growth factors. Results also showed differences in the kinetics of activation of JNK and ERK. The maximal activation of JNK was obseved at 20 min whereas ERKs at 10 min. Differences in the kinetics and the activation by IL-1β and FGF-2 indicate that ERK and JNK are distinct pathways. Although IL-1 had been shown to activate ERK in human lung fibroblasts [Guesdon et al., 1993], monocytes [Ahlers et al., 1994], mesangial cells [Wilmer et al., 1997], and both ERK and JNK in rabbit articular chondrocytes [Scherle et al., 1997]; however, our data showed a small effect on the ERK activation by IL-1β in human and rat osteoblastic cells. These differences in the activation of ERK and JNK pathways may be due to differences in the cell types and/or species.

The accumulated data suggest that IL-1β may affect osteoblast function by activating MAP kinases preferentially JNK, which can then regulate gene transcription. IL-1β activation of the JNK pathway in osteoblasts most likely plays an important role in mediating its effects on bone remodeling, in part due to the effects of MAP kinases on the transcription factor AP-1, which is composed of homo- and heterodimer of Jun and Fos proteins. ERK phosphorylates and increases the activity of the transcription factor, TCF/Elk-1, which is required for enhanced transcription of the c-fos

gene [Minden et al., 1994]. JNK phosphorylates c-Jun at two critical N-terminals, Ser63 and Ser73, which enhances the ability of c-Jun to activate transcription [Su and Karin, 1996; Minden et al., 1994]. JNK also phosphorylates activating transcription factor-2 (ATF-2), which binds to the c-Jun promoter and up-regulates its transcription [Su and Karin, 1996]. Together ERK and JNK pathways lead to increased AP-1 activity. Increased AP-1 leads to upregulation of matrix metalloproteinases (MMPs) and suppression of tissue inhibitor of metalloproteinases (TIMP), resulting in an increased degradation of extracellular bone matrix [Tsuji et al., 1996]. This may be the one of the mechanisms by which IL-1β stimulates bone resorption and involves the pathogenesis of osteoporosis. In summary, our data have clearly demonstrated that IL-1β and FGF-2 differentially activate MAP kinase pathways (JNK and ERK) and provide additional mechanisms by which IL-1β and FGF-2 regulate osteoblast function and gene expression.

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