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Interleukin-1 Regulates FGF-2 mRNA and Localization of FGF-2 Protein in Human Osteoblasts

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Interleukin-1 (IL-1) and basic fibroblast growth factor (FGF-2) are potent stimulators of osteoclast formation. However, the role of FGF-2 in the responses to IL-1 in bone has not been reported. We examined the effect of IL-1 on FGF-2 mRNA and protein expression in human osteosarcoma MG-63 osteoblasts, normal human osteoblasts (NHOB), and osteoblasts from osteoarthritic patients (F2 and F13). IL-1 increased FGF-2 mRNA expression in osteoblasts within 1.5 to 3 h. Multiple FGF-2 protein isoforms were expressed in human osteoblasts. Twenty-four hours of treatment of MG-63 and NHOB cells with IL-1 increased the high-molecular-weight (HMW, 22/24 kDa) and low-molecular-weight (LMW, 18 kDa) FGF-2 proteins intracellularly. In contrast, IL-1 preferentially increased the LMW protein signal intracellularly as well as on the cell surface of F2 and F13 osteoblasts. We conclude that IL-1 is a major stimulator of FGF-2 expression in human osteoblasts. Furthermore, selective increases in the exportable LMW protein in osteoblasts from osteoarthritic patients may be of clinical relevance.

Key Words: **interleukin-1; FGF-2 mRNA; FGF-2 protein isoforms; osteoblasts; osteoarthritis.**

Members of the fibroblast growth factor (FGFs) family and their tyrosine kinase receptors (FGFRs) have been shown to be important in both normal bone remodeling and in pathologic disorders of bone (1–4). There are 23 distinct FGFs (4), of these, FGF-2 has been most highly studied in bone. FGF-2 that is produced by osteoblasts (5) and stored in bone matrix, is a potent mitogen for osteoblasts (6–9). Chronic FGF-2 treatment inhibits bone formation *in vitro* (10, 11). However, when administered intermittently, it stimulates bone formation *in vivo* (12). The development of FGF-2 null mice has enabled us to examine the role of endogenous FGF-2 in bone formation (13). We recently reported that disruption of the FGF-2 gene in mice resulted in decreased bone mass associated with decreased trabecular number and decreased bone formation (14). In other studies, we showed that FGF-2 increased bone resorption in mouse calvarial cultures (15) and increased osteoclast formation in murine bone marrow cultures (16). Thus, FGF-2 may function as a coupling factor since it is a regulator of both osteoblast and osteoclast formation.

Interleukin-1 (IL-1) is a proinflammatory cytokine which is a major stimulator of bone resorption (17) and osteoclast formation (18, 19). Studies have shown that macrophages, osteoblasts and osteoclasts, contribute to production of local bone IL-1 (17). Although IL-1α and IL-1β are encoded by separate genes, they have similarity of action (20). Studies have shown that some of the responses to IL-1 in bone are due to induction of mediators such as IL-6 and PGE_2 (20). Similar to IL-1, FGF-2 is a potent stimulator of both IL-6 (21) and PGE_2 (15) production by osteoblasts. Furthermore, recent studies have shown that both FGF-2 (22) and IL-1 (23) are potent stimulators of receptor activator of NF-kappa B ligand (RANKL) which is required for osteoclast formation. In terms of physiological relevance, IL-1 has been implicated in osteoporosis (24–26), inflammatory disorders such as periodontal disease (27) and the joint inflammation of rheumatoid arthritis (28). Interestingly, recent studies suggest that endogenous FGF-2 in joint fluid may contribute to the joint destruction in rheumatoid arthritis patients (29).

We previously reported that stimulators of bone resorption and osteoclast formation such as parathyroid hormone (PTH) (30) and prostaglandins (PGs) (31) regulated FGF-2 mRNA, as well as FGF-2 protein production in rodent osteoblasts. However, the effect of IL-1 to regulate FGF-2 gene expression in osteoblastic cells has not been investigated. We therefore, examined the ability of IL-1 to regulate FGF-2 expression in human

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osteosarcoma tumor cell line (MG-63) (32), transformed human osteoblastic cells (HOBIT), (33), osteoblasts from two female patients with osteoarthritis (F2, F13) and normal human osteoblasts (NHOB).

In this study, we report for the first time that IL-1 not only increased FGF-2 mRNA but also regulated the isoform profile of FGF-2 protein expression in human osteoblasts as well as the localization of FGF-2 protein. Our observation that the 18-kDa isoform was exported from F2 and F13 osteoblasts may be an important example of translation regulation of FGF-2 gene expression in physiologically relevant human cells. These data suggest that endogenous FGF-2 may be important in the biologic responses to IL-1 in bone.

MATERIALS AND METHODS

Materials. Recombinant human Interleukin-1 α and β were purchased from Endogen (Cambridge, MA). Dulbecco's modified Eagle's medium (DMEM), HAM F-12 medium, bovine serum albumin, phorbol myristate acetate (PMA), forskolin (FSK), and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Phosphoascorbic acid (L-ascorbic acid phosphate magnesium salt) was obtained from Wako Pure Chemical Industries (Osaka, Japan).

Cell cultures. Human osteosarcoma cells (MG-63), transformed human osteoblasts (HOBIT), osteoblasts from patients with osteoarthritis (F2, F13), and osteoblasts from normal subjects (NHOB) were utilized in these studies. Human osteosarcoma MG-63 cells (32) were purchased from the American Type Culture Collection (CRL-1427; Rockville, MD). They were plated in 6-well dishes (Costar, Cambridge, MA) at a density of 5000 cells/cm^2 and grown to confluence over 6 days in DMEM without phenol red containing 10% heat-activated fetal bovine serum (FBS; Life Technologies, Grand Island, NY), penicillin (1000 U/ml), and streptomycin (50 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere. Media were changed every 3 days. Before treatment, MG-63 cells were cultured in serum-free DMEM with 0.1% bovine serum albumin for 24 h except as noted. All treatment groups were controlled for the vehicle of test material.

HOBIT cells were obtained from Dr B. Lawrence Riggs (Mayo Clinic and Mayo Foundation, Rochester, MN) (33). They were plated in six-well dishes at a density of 5000 cells/cm^2 and grown for 3 weeks in DMEM and HAM F-12 media with 10% heat-activated FBS, penicillin (1000 U/ml), and streptomycin (50 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere. Media were changed every 3 days. Cells were grown for about 3 weeks. HOBIT cells were utilized when they were about 80% confluent and were not serum deprived before treatment. Because of the slow growth of these cells, only limited studies were conducted in HOBIT cells. All treatment groups were controlled for the vehicle of test material. Because of the slow growth of these cells, only limited studies were conducted.

Human osteoblasts obtained from two female subjects with osteoarthritis were kindly provided by Dr. Yoshiya Tanaka (University of Occupational and Environmental Health, Fukuoka, Japan). Metaphyseal trabecular bone was obtained from the proximal femur of two female osteoarthritic patients during total hip arthroplasty. They were designated as F2 and F13. Bone fragments were cut into small pieces, washed and cultured in DMEM with 10% FBS. When cells were confluent, explants were removed and the cells were replated at 5000 cells/cm^2 and cultured to either 80% confluence or confluence before treatment.

Normal human osteoblasts (NHOB) were purchased from Clonetics (Walkersville, MD). They were plated in 100-mm dishes at a density of 5000 cells/cm^2 and grown to confluence over 6 days in DMEM and HAM F-12 media with 10% heat-activated FBS, penicillin (1000 U/ml), and streptomycin (50 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere.

cDNA probes. A 0.4-kb mouse FGF-2 cDNA (34) was a gift from Dr. Gail Martin (University of California, San Francisco, CA). A rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe was utilized as a control (35).

Measurement of mRNA levels. Total RNA was extracted from cells by the method of Chomczynski and Sacchi (36). Cells were scraped in 4 M guanidinium thiocyanate and extracted with phenol/chloroform isoamyl alcohol (24:1) and total RNA precipitated with isopropanol. For Northern analysis, 12.5 μg of total RNA was denatured and fractionated on a 1% agarose/2.2 M formaldehyde gel, transferred to nylon membrane by positive pressure and fixed to the filter by UV irradiation (Stratalinker) (37). After a 4-h prehybridization, filters were hybridized overnight with a random primer d-CTP-labeled [^{32}P]cDNA probe for the mRNA of interest overnight. Bands were normalized to GAPDH. Signals were quantitated by densitometry and normalized to the corresponding value for GAPDH.

Western blot analysis. FGF-2 protein levels in osteoblastic cells were determined by Western blot analysis (38–40). Cells from subconfluent or confluent 100-mm dishes were harvested in 1 ml of lysis buffer (62.5 mM EDTA pH 8.0, 50 mM Tris, pH 7.5, 0.4% deoxycholate (Sigma), 1% NP-40 (Calbiochem, La Jolla, CA) containing protease inhibitors [0.01 mM phenylmethylsulfonyl fluoride (PMSF, Calbiochem), 10 ng/ml aprotinin (Sigma), 10 ng/ml leupeptin, (ICN, Costa Mesa, CA), 10 ng/ml pepstatin (ICN), and 50 μM AEBSF (Sigma)]. The cell extract was clarified by microfuge centrifugation (14,000 rpm, 15 min at 4°C) and heparin–Sepharose (HS) (CL-6B, Pharmacia, Piscataway, NJ) was added and the mixture incubated, rocking at 4°C for 2 h. The mixture was washed 3× with lysis buffer, 3× with HS-wash buffer (20 mM Tris, pH 7.4, 5 mM EDTA, 2 mM EGTA, plus protease inhibitor) containing 0.5 M NaCl and 3× with HS-wash buffer containing 1.0 M NaCl. Proteins that remained bound to HS were eluted directly into SDS gel sample buffer and resolved by 12% SDS–polyacrylamide gel electrophoresis (PAGE). For Western blot analysis, proteins were transferred to nitrocellulose (PROTRAN, pore 0.45 μm Schleicher and Schuell, Keene, NC) in cold buffer containing 25 mM 3-[dimethyl(hydroxymethyl)methylamino]-2-hydroxypropane sulfonic acid (AMPSO, Research Organics Inc., Cleveland, OH), pH 9.5, 20% methanol for 90 min at continuous 0.4 amps. Transfers were blocked at room temperature for 1 h in buffer containing 10 mM Tris 7.5, 150 mM NaCl, 5 mM NaN_3, 0.35% polyoxyethylene–sorbitan monolaurate (Tween 20, Sigma), and 5% nonfat dry milk (Carnation Co., Los Angeles, CA). Transfers were incubated with anti-FGF-2 immune serum (1:1200) in blocking buffer at 4°C for 16 h, washed at room temperature with 1 liter (10 changes) of buffer containing 150 mM NaCl, 500 mM sodium phosphate, pH 7.4, 5 mM NaN_3 and 0.05% Tween 20. Transfers were then incubated for 1 h in blocking buffer containing 15 μCi ^{125}I-protein A (ICN Biochemicals,) and washed with 1 liter of buffer as described above. Signal was visualized by autoradiography using RX Fuji Medical X-ray film and/or by scanning the blot by phospho-Imager.

Statistical analysis. Means of groups from Northern analysis were compared using analysis of variance (ANOVA) and the Bonferroni post hoc test when ANOVA demonstrated significant differences.

RESULTS

IL-1 Regulates FGF-2 mRNA Expression in MG-63 Cells

Northern blot analysis showed that MG-63 cells expressed multiple FGF-2 mRNA transcripts in control cultures including major bands of 4 and 7 kb (Fig. 1). Time course studies showed that IL-1 (10 ng/ml) in-

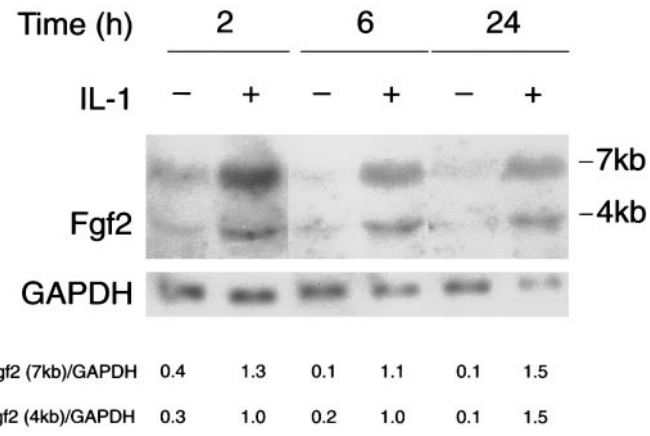

FIG. 1. Time course of the effects of IL-1 (10 ng/ml) on FGF-2 mRNA expression in MG-63 osteoblasts. Cells were serum deprived for 24 h and treated with or without effector. RNA was extracted at each time point for Northern blot analysis as described under Materials and Methods.

creased the 7-kb transcript by 3-fold at 2 h, 11-fold at 6 h, and 15-fold at 24 h. The 4-kb transcript was increased 3-fold at 2 h, 5-fold at 6 h, and 15-fold at 24 h. Previous studies showed that both PMA an activator of the protein kinase C (PKC) signaling pathway and FSK, an activator of the protein kinase A (PKA) pathway regulated FGF-2 expression in human adrenal cells (39). We therefore examined whether PMA or FSK mimicked the effect of IL-1 to regulate FGF-2 mRNA expression. As shown in Fig. 2, treatment of MG-63 cells with PMA (1 μM) for 3 h caused a 2- and 5-fold increase in the 7- and 4-kb FGF-2 mRNA transcripts, respectively. Furthermore, the stimulatory effect of PMA on the 7-kb transcript was additive to IL-1. Treatment of MG-63 cells with FSK (10 μM) for 3 h caused a 4- and 5-fold increase in the 7- and 4-kb FGF-2 mRNA, respectively, but there was no additive effect of IL-1 (Fig. 2).

IL-1 Regulates FGF-2 mRNA Expression in HOBIT Cells

HOBIT cells expressed multiple FGF-2 mRNA transcripts in control cultures including major bands of 4 and 7 kb. A representative time course study is shown in Fig. 3. IL-1 (10 ng/ml) increased FGF-2 mRNA in HOBIT cells within 1.5 h and this was sustained for 24 h. Maximum increase was observed between 3 and 12 h.

IL-1 Regulates FGF-2 mRNA Expression in Osteoblasts (F13) from an Osteoarthritic Patient

We examined the time course of the effects of IL-1 treatment on FGF-2 mRNA expression in human osteoblasts (F13) from an osteoarthritic patient (Fig. 4). There was a 2.5-fold increase in the 7 kb FGF-2 mRNA and a 2-fold increase in the 4-kb transcripts within 3 h which was sustained for 24 h. Maximum increase in FGF-2 mRNA transcripts were seen at 6 h at which time IL-1 caused a 5.5- and 7-fold increase in the 7- and 4-kb mRNA transcripts, respectively. Three hours of treatment with PMA (1 μM) or FSK (10 μM) also increased the 7-kb FGF-2 mRNA expression approximately 2-fold in these cells.

IL-1 Regulates FGF-2 Protein Isoform Levels in Human Osteoblasts

We next compared the effect of treatment with IL-1β (10 ng/ml) and IL-1α (10 ng/ml) on FGF-2 protein levels in confluent MG-63 osteoblasts, as well as subconfluent F2 and F13 osteoblasts from two different patients with osteoarthritis (Fig. 5). Western blot analysis showed that MG-63 cells expressed high-molecular-weight (HMW)22/24-kDa proteins as a doublet and a low molecular weight (LMW) 18-kDa isoform of FGF-2 protein in control cultures. Sixteen hours of treatment, with either IL-1β or IL-1α, caused a 5.4-fold increase in the 22/24-kDa proteins. IL-1β caused a 2-fold increase in the 18-kDa signal while IL-1α caused a 3.9-fold increase in the 18-kDa signal. In F2 and F13 osteoblasts, there were very low levels of the HMW 22/24-kDa FGF-2 proteins in vehicle treated cultures (Fig. 5) and there was minimal regulation by IL-1. In contrast, IL-1β and IL-1α caused a 2.9-fold increase in the 18-kDa signal in F2

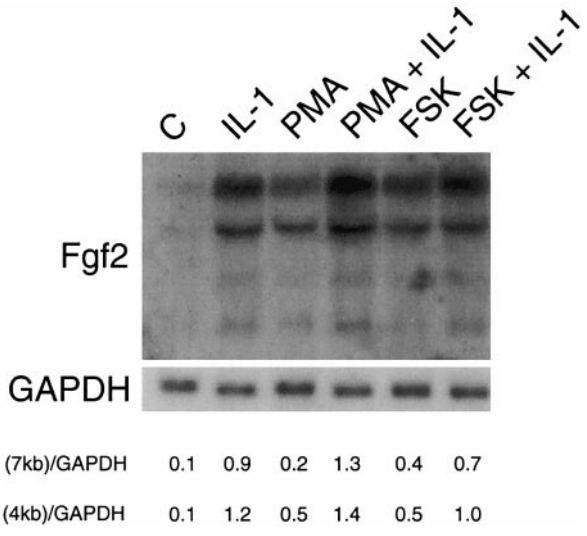

FIG. 2. Effect of 3 h of treatment with IL-1 alone or in combination with PMA (1 μM) or FSK (10 μM) on FGF-2 mRNA levels in MG-63 cells. Cells were grown to confluence and then serum-deprived in the presence of BSA (1 mg/ml) for 24 h. After 24 h, cultures were treated for an additional 3 h with effectors. Total RNA was extracted for Northern blot analysis as described under Materials and Methods.

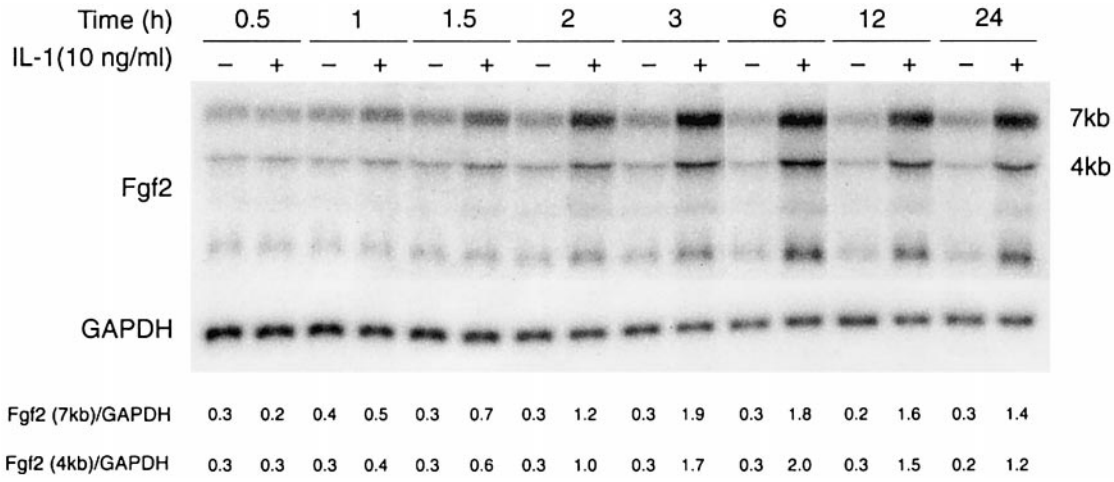

FIG. 3. Time course of the effects of IL-1 (10 ng/ml) on FGF-2 mRNA expression in HOBIT cells. Confluent cells were treated with or without effectors in the presence of 10% serum. RNA was extracted at each time point for Northern blot analysis as described under Materials and Methods.

osteoblasts. IL-1β caused a 3.8-fold and IL-1α caused a 2-fold increase in the 18-kDa signal in F13 osteoblasts.

To ensure that the degree of confluence did not alter the FGF-2 protein profile in F13 osteoblasts, we conducted additional studies and compared the FGF-2 protein isoform profile of confluent osteoblasts from osteoarthritic patient (F13) with that from confluent osteoblasts from normal patients (NHOB) following 16 h treatment with IL-1α. As shown in Fig. 6, NHOB cell expressed all isoforms of FGF-2 proteins in control cultures. IL-1α (10 ng/ml) caused 2- and 3-fold increases in the HMW 24- and 22-kDa proteins respectively and a 3.9-fold increase in the 18-kDa isoform. IL-1α (10 ng/ml) caused a 1.3-fold increase in the 22-kDa protein and a 1.9-fold increase in the 18-kDa isoform in F13 osteoblasts. Thus, IL-1 significantly increased the relative steady state level of FGF-2 protein detected in primary osteoblast cultures originating from normal as well as patients manifesting an osteoarthritic phenotype.

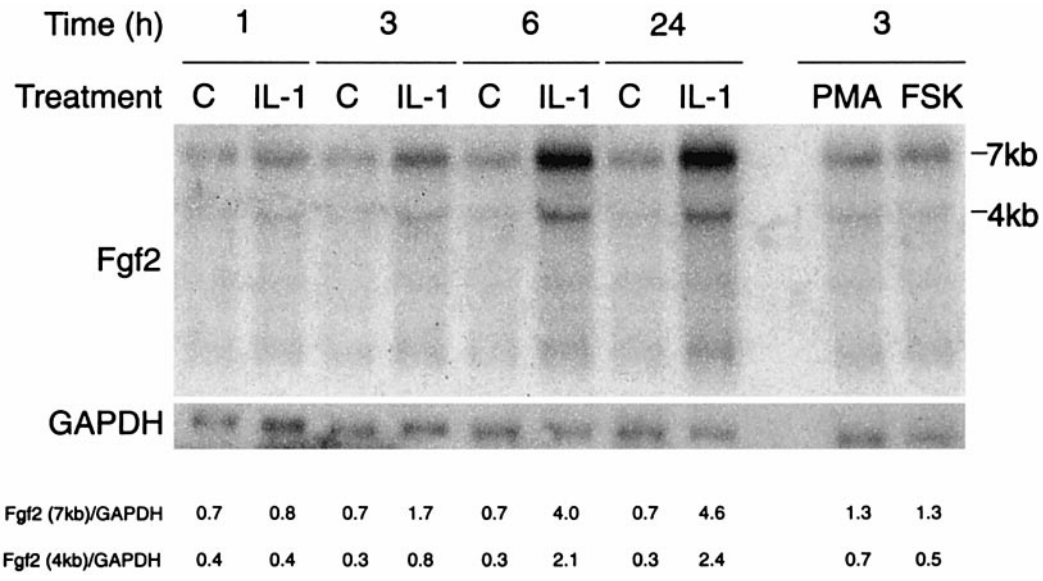

FIG. 4. Time course of the effects of IL-1 (10 ng/ml) on FGF-2 mRNA expression in osteoblasts from osteoarthritic patient (F13) cells. Effect of 3 h of treatment with PMA (1 μM) and FSK (10 μM) on FGF-2 mRNA is shown. Confluent cells were treated with or without effectors in the presence of 10% serum. RNA was extracted at each time point for Northern blot analysis as described under Materials and Methods.

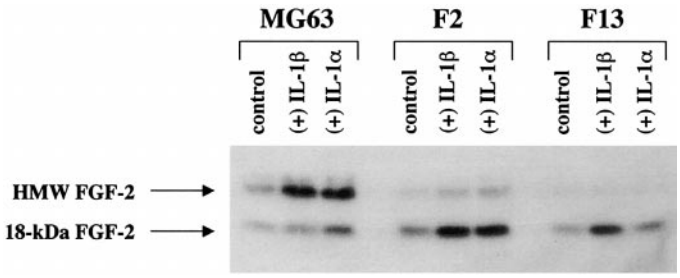

FIG. 5. Effect of IL-1β (10 ng/ml) and IL-1α (10 ng/ml) on FGF-2 protein accumulation in MG-63 cells and osteoblasts from patients with osteoarthritis (F2 and F13). Cells were grown to confluence in 100-mm dishes. After 24 h serum deprivation, cells were treated with effectors in the absence serum. Western analysis for FGF-2 protein was performed as described under Materials and Methods.

IL-1 Differentially Regulates FGF-2 Protein Isoform Localization in Osteoblasts from Osteoarthritic Patients

Since IL-1 preferentially increased the exportable 18-kDa protein isoform in F2 and F13 osteoblasts from osteoarthritic patients, we examined whether IL-1 modulated the localization of this FGF-2 protein isoform. A representative immunoblot of F13 osteoblasts treated with vehicle or IL-1 for 16 h is shown in Fig. 7. Both the 22/24-kDa and the 18-kDa isoforms were detected intracellularly; however, the 22/24-kDa signal was much weaker than the 18-kDa signal. There was a 1.5-fold increase in the 22/24-kDa and a 1.4-fold increase in the 18-kDa intracellular signals following 16 h of treatment with IL-1. Interestingly, the 22/24-kDa isoform was not detected on the cell surface in either vehicle or IL-1-treated cultures. In contrast, the 18-kDa isoform was detected on the cell surface and following treatment with IL-1α (16 h) was increased 2.3-fold.

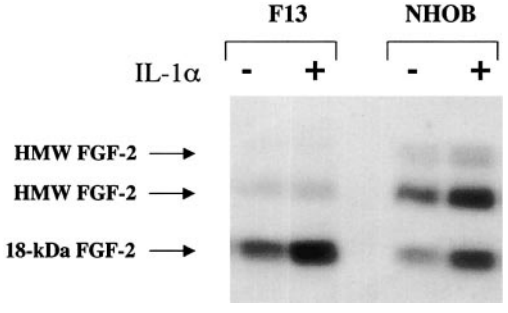

FIG. 6. Comparison of the effect of IL-1α (10 ng/ml) on FGF-2 protein isoforms in osteoblasts from a patient with osteoarthritis (F13) and normal primary osteoblasts (NHOB). Cells were grown to confluence in 100-mm dishes. After 24 h serum deprivation, cells were treated with effectors in the absence of serum for 16 h. Western analysis for FGF-2 protein was performed as described under Materials and Methods.

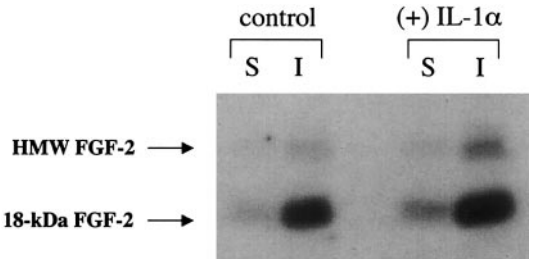

FIG. 7. Effect of treatment with IL-1α (10 ng/ml) on cell surface (S) and intracellular (I) FGF-2 protein accumulation in F13 osteoblasts obtained from a patient with osteoarthritis. Cells were treated with IL-1 for 16 h. Immunoblot analysis was performed as described under Materials and Methods.

DISCUSSION

This study demonstrates that multiple FGF-2 mRNA transcripts are expressed in human osteoblasts. We examined IL-1 regulation of FGF-2 mRNA expression in MG-63 cells which were derived from a patient with osteosarcoma (32). We also examined the effect of IL-1 on FGF-2 mRNA in HOBIT cells, a transformed clonal cell line derived from normal human osteoblast-like cells (33) and primary osteoblasts derived from patients with osteoarthritis. In all human osteoblast cell lines as well as primary human osteoblasts from osteoarthritic patients, IL-1 treatment increased FGF-2 mRNA within 2–3 h and this was sustained for 24 h.

In MG-63 cells, PMA an activator of the PKC pathway and FSK an activator of the PKA/cAMP pathway which were previously shown to regulate FGF-2 expression in osteoblasts (31, 30) caused a small increase in FGF-2 mRNA expression. PMA and IL-1 were additive to increase FGF-2 mRNA, in MG-63 cells. In contrast, there was no additive response of IL-1 and FSK. PMA and FSK also increased FGF-2 mRNA in F13 osteoblasts. These results suggest that IL-1 regulation of FGF-2 gene expression involve multiple signaling pathways.

Multiple isoforms of FGF-2 protein were produced in human osteoblasts examined in this study and is consistent with earlier studies showing that the human FGF-2 gene encoded multiple FGF-2 polypeptides (38). Previous studies showed that initiation of translation at CUG2/CUG3 codons located 5′ to the AUG codon accounted for the high-molecular-weight (HMW-22 and 24 kDa) nuclear FGF-2 proteins, whereas initiation at the AUG codon was used for initiation of translation of the 18-kDa protein (38). Earlier studies also showed that alternative translation determined cytoplasmic or nuclear localization of FGF-2 (40, 41) and that nuclear forms of FGF-2 could regulate gene transcription (42). Other studies have shown that selective expression of the various isoforms of FGF-2 modulated cell phenotype (43).

In this study, IL-1 treatment increased FGF-2 protein levels. Western blot analysis (Figs. 5, 6, and 7) showed clearly and reproducibly that IL-1α and IL-1β significantly increased the relative steady state level of FGF-2 protein isoforms detected from the continuously growing human osteosarcoma cell line MG-63, primary osteoblasts originating from normal patients (NHOB) as well as osteoblasts from patients manifesting an osteoarthritic phenotype (F2, F13). However, there were interesting variations in FGF-2 isoform accumulation in response to IL-1 treatment in these osteoblasts. We observed that MG-63 and NHOBs expressed all isoforms with stoichiometrically similar levels of translation initiation from CUG2/CUG3 for the 22/24-kDa isoforms and the AUG for the 18-kDa isoform. However, both F2 and F13 osteoblasts consistently expressed much higher steady state levels of 18-kDa FGF-2 protein relative to any HMW isoform. Under the steady-state conditions examined in these experiments, 18-kDa FGF-2 protein was detected intracellularly and on the cell surface of F2 and F13 osteoblasts. HMW protein was detected intracellularly but not on the surface of F2 and F13 osteoblasts. Cell surface FGF-2 detected following treatment with IL-1α for 16 h was increased 10-fold. Although this represents only about 15% of total signal detected, these results are significant when compared with studies in COS cells where the rate of FGF-2 export was rather slow ($T_{1/2}$ about 6–8 h) (44). Previous studies in COS cells have shown that the 18-kDa isoform of FGF-2 protein can be exported to the cell surface (40). The cell surface FGF-2 signal represents protein trafficking through the "export" (non-ER/Golgi exocytic) pathway (40) and should be considered to be in a bioavailable compartment, at least with respect to plasma membrane localized receptor(s) (41).

Our observation that the 18-kDa isoform was exported from F2 and F13 osteoblasts, may be an important example of translation regulation of FGF-2 gene expression in physiologically relevant human cells. These observations are particularly interesting since previous studies showed that synovial tissue obtained from patients with rheumatoid arthritis, degenerative joint disease or following trauma expressed FGF-2 (45) protein and that FGF-2 modulated cellular responses to IL-1 (46). Exogenous FGF-2 treatment was shown to enhance the effect of IL-1 to synthesize collagenase and neutral proteases by chondrocytes (46) and to produce a synergistic increase in prostaglandin production (47). It is possible that increased export of FGF-2 unto the osteoblast cell surface could potentiate some of the biologic responses to IL-1 in bone.

In summary, we have shown that IL-1 not only increased FGF-2 mRNA but also regulated the isoform profile of FGF-2 protein expression in human osteoblasts. These data suggest that endogenous FGF-2 may be an important mediator of some of the biologic responses to IL-1 in bone. We recently reported on the bone phenotype in mice with disruption of the FGF-2 gene (14). These mice will be a useful model to study the biological relevance of IL-1 regulation of FGF-2 protein expression in osteoblasts.

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