Transcriptional Regulation of the Osterix (Osx, Sp7) Promoter by Tumor Necrosis Factor Identifies Disparate Effects of Mitogen-activated Protein Kinase and NFκB Pathways*

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Osteoblast (OB) differentiation is suppressed by tumor necrosis factor-α (TNF-α), an inflammatory stimulus that is elevated in arthritis and menopause. Because OB differentiation requires the expression of the transcription factor osterix (Osx), we investigated TNF effects on Osx. TNF inhibited Osx mRNA in pre-osteoblastic cells without affecting Osx mRNA half-life. Inhibition was independent of new protein synthesis. Analysis of the Osx promoter revealed two transcription start sites that direct the expression of an abundant mRNA (Osx1) and an alternatively spliced mRNA (Osx2). Promoter fragments driving the expression of luciferase were constructed to identify TNF regulatory sequences. Two independent promoters were identified upstream of each transcription start site. TNF potently inhibited transcription of both promoters. Deletion and mutational analysis identified a TNF-responsive region proximal to the Osx2 start site that retained responsiveness when inserted upstream of a heterologous promoter. The TNF response region was a major binding site for nuclear proteins, although TNF did not change binding at the site. The roles of MAPK and NFκB were investigated as signal mediators of TNF.

Inhibitors of MEK1 and ERK1, but not of JNK or p38 kinase, abrogated TNF inhibition of Osx mRNA and promoter activity. TNF action was not prevented by blockade of NFκB nuclear entry. The forced expression of high levels of NFκB uncovered a proximal promoter enhancer; however, this site was not activated by TNF. The inhibitory effect of TNF on Osx expression may decrease OB differentiation in arthritis and osteoporosis.

Osteoblasts (OBs) are derived from pluripotent precursor cells of mesenchymal origin that are capable of differentiation to chondrocytes, myocytes, adipocytes, or fibroblasts. Bone formation in the embryo and remodeling in the adult require that a sufficient number of precursor cells differentiate to functional OBs. New OBs are continuously required for the synthesis of bone matrix and replacement of cells becoming osteocytes or undergoing apoptosis. A coordinated expression of transcription factors determines the commitment of precursor cells toward the OB phenotype under the control of autocrine, paracrine, and hormonal stimuli. Two of these transcription factors, RUNX2 (Cbfa1/AML3/Pebp2A) and Osx, are required for differentiation to the OB lineage. In mice, RUNX2 gene knock-out causes a lethal mutation with a cartilaginous skeleton. RUNX2 is presumed to function as an organizer on promoters of skeletal-specific genes (2). A phenotype similar to the RUNX2 knock-out is observed with knock-out of Osx. Here the arrest in development occurs slightly later but also results in a cartilaginous skeleton (3). In addition, Osx induces OB differentiation of dispersed embryonic cells (4). RUNX2 is expressed in Osx knock-outs, suggesting that Osx functions downstream of RUNX2 in the differentiation pathway.

Differentiation of precursor cells to OBs in adult bone is impaired by inflammatory stimuli. In rheumatoid arthritis, estrogen deficiency, and aging, there is an increased expression of cytokines, including tumor necrosis factor-α (TNF-α). In adult bone, inflammatory cytokines blunt the formation rate of new bone in the face of increased resorption, contributing to net bone loss (5–11). Cytokines could interfere with the expression of factors required for OB differentiation.

We have previously shown that TNF inhibits osteoblast differentiation at the stage of precursor cell commitment to the OB lineage (6). Osteoblast precursors, including fetal calvaria cells, murine marrow stromal cells, and the clonal pre-osteoblastic cell line MC3T3, fail to differentiate in the presence of TNF. These models of osteoblast progenitors uniformly show enhanced sensitivity to TNF blockade of differentiation at an early stage in culture when the key transcription factors RUNX2 and Osx are required. An inhibitory effect of TNF on the RUNX2 promoter that could contribute to decreased expression and differentiation of cells has been described previously (12). This effect of TNF is isoform-specific, inhibiting the osteoblastic MASNS RUNX2 isoform 50% and the more ubiquitously expressed MRIPV isoform >90%. These results suggest that TNF may have additional targets.

Here we present evidence that TNF is a potent inhibitor of Osx expression. In addition, we have evaluated the structure and regulation of the Osx promoter and report transcriptional regulation by TNF at a discrete site via a mitogen-activated protein kinase (MAPK) signal.

MATERIALS AND METHODS

Reagents—MC3T3-E1 (clone 14) mouse pre-osteoblast cells were provided by Dr. Renny Franceschi (University of Michigan). C3H10T1/2 cells were obtained from the American Type Culture Collection (Manassas, VA). Human TNF-α was purchased from PeproTech (Rocky Hill, NJ). Real-time PCR was done using the Bio-Rad iCycler. SYBR Green was obtained from Bio-Rad. MAPK inhibitors PD98059 and SB203580 were obtained from Calbiochem. SP600125
was purchased from Tocris Cookson (Ellisville, MO). Minimal essential medium was purchased from Invitrogen and fetal bovine serum from Hyclone (Logan, UT). Other reagents were obtained from Sigma.

Cell Treatment and RNA Harvest—MC3T3-E1 cells were plated on day 0 at 7.4 x 10^5 cells/150-mm plate in minimal essential medium + 10% fetal bovine serum (medium). On day 1, medium was replaced with differentiating medium (minimal essential medium + 10% fetal bovine serum + 50 μg/ml l-ascorbate). On day 2, TNF-α was added in the doses indicated for each experiment. The half-life of Osx mRNA was measured in MC3T3-E1 cells plated on day 0 at 4.4 x 10^5 cells/well in 6-well plates in medium. Actinomycin D (0.5 μg/ml) was added 2 h prior to TNF-α, and RNA was obtained at the time points indicated under “Results.” For experiments using cycloheximide, the same protocol was followed as for actinomycin D, except 5 μg/ml cycloheximide was used. RNA was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA). The addition of MAPK chemical inhibitors was done 2 h prior to TNF treatment. Transient transfection of dominant negative MEK1 or ERK1 was done with the Osx promoter reporter construct followed to TNF treatment. Transient transfection of dominant negative MEK1 was used. The addition of MAPK chemical inhibitors was done 2 h prior to TNF (10 ng/ml); D, new protein synthesis is not required for TNF inhibition of Osx mRNA.

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FIGURE 1. TNF inhibits Osx mRNA. MC3T3 cells were treated with human TNF-α, and total RNA was isolated at the times indicated. mRNA was measured by real-time RT-PCR and is expressed in relative units. A, time course of TNF action (10 ng/ml). B, dose response effect of TNF on day 2 of culture. C, Osx mRNA stability is not changed by TNF. Cells were treated with actinomycin D (0.5 μg/ml) 2 h prior to the addition of TNF. mRNA stability is shown as % mRNA remaining after the addition of TNF (10 ng/ml); D, new protein synthesis is not required for TNF inhibition of Osx mRNA. Cycloheximide (CHX) (5 μg/ml) was added 2 h prior to TNF (10 ng/ml). Osx mRNA was measured 24 h after the addition of TNF. * p < 0.05 compared with control (A and D) or compared with the matched group (D). Differences in C were not significant.
Transfection—The C3H10T1/2 cells (n = 3 wells/group) were plated at a density of 7 × 10^4 cells/well in 12-well tissue culture plates (Corning, NY). After 24 h, the cells were transfected with a mixture of Superfect transfection reagent (Qiagen, Valencia, CA), medium, promoter reporter, and pRL-TK control vector (Promega, Madison, WI). 48 h after transfection, cells were harvested and assayed using firefly luciferase and Renilla luciferase substrates in the Dual Luciferase assay system (Promega, Madison, WI). Luciferase values were normalized to Renilla luciferase data to correct for variation in transcription efficiency.

Rapid Amplification of 5’-cDNA Ends—The 5’-end sequence of the alternatively spliced exon was obtained using BD SMART RACE cDNA amplification kit (BD Biosciences) following the manufacturer's instructions. The primer used was 5'-GAG CTT CTT CCT CAA GCA GAG AGG ACG CCA TCC TCG TCG A-3’. The PCR products were cloned into the pCR-TOPO vector using the TOPO TA cloning kit (Invitrogen) and analyzed on 6% (w/v) denaturing polyacrylamide gels. The gels were dried and analyzed in a phosphorimaging device.

Electrophoretic Mobility Shift Assay (EMSA)—Electrophoretic mobility shifts were done as previously described (12, 13).

Statistical Analysis—ANOVA was used to determine a statistical difference between multiple groups. Multiple comparisons between individual groups were done by the method of Tukey. Comparisons between any one group and a common control were done by the method of Dunnet. Analyses were done using Prism software, (GraphPad, San Diego, CA).

RESULTS

TNF Inhibits Osx Steady State mRNA—Osx expression was observed by day 2 of culture in MC3T3 cells. The effect of TNF was studied using doses previously shown to inhibit osteoblast differentiation in MC3T3, bone marrow stromal, and fetal rat calvaria pre-osteoblasts (6, 14). Fig. 1A shows that treatment with 10 ng/ml TNF on day 2 of culture inhibited Osx steady state mRNA. TNF inhibited Osx mRNA 50% by 4 h, 85% by 8 h, and 95% by 24 h compared with levels in the control cultures. Fig. 1B shows that the inhibitory effect of TNF was dose-dependent with 50% inhibition of mRNA occurring at 0.75 ng/ml. This IC50 is similar to that reported for TNF inhibition of osteoblast differentiation (6). To determine whether TNF decreased Osx by destabilization of mRNA, MC3T3 cells were treated with actinomycin D prior to the addition of TNF to stop RNA synthesis. The half-life of the Osx mRNA was then measured over 14 h using real-time RT-PCR. TNF did not decrease the steady state mRNA. TNF further inhibited the Osx mRNA level in the presence of cycloheximide treatment.

Cloning of the Osx Promoter and Determination of Transcriptional Start Sites—A 1357-bp fragment of the proximal promoter including a portion of 5’-untranslated RNA was amplified from a murine F-factor-based bacterial artificial chromosome containing genomic Osx, sequenced for confirmation, and cloned into the KpnI and XhoI sites of the pGL3 Basic-luciferase reporter. A map of homologous transcription factor binding sites was generated using MatInspector to assess potential regulatory sites (15). Fig. 2 shows that the promoter contains putative homologous binding sites for factors known to influence differentiation of pluripotent precursor cells to the osteoblast, chondrocyte, adipocyte, or myoblast lineage. These included Mys, AML-1, RUNX2, cEBP, Msx, DLX, and NFκB core consensus sequences. The Osx promoter fragment also included 14 contiguous repeats of a Mys-binding site.
The transcriptional start site of the Osx promoter was determined by 5' RACE. Fig. 3A shows that 5'-RACE yielded two bands of 100 and 300 kb size, suggesting two potential start sites. (also identified in Fig. 2). These sites were confirmed by primer extension (not shown). Amplification using unique 5' primers and a common 3' primer yielded two mRNA species designated Osx1 and Osx2 (Fig. 3B). Sequencing revealed that the more abundant Osx1 corresponded to the expected mRNA as reported previously for murine Osx2. Osx2 is a slightly larger species in harmony with the highly homologous human Osx1. The less abundant Osx2 includes alternatively spliced exons 1 and 2. Fig. 3C maps the genomic Osx structure as deduced from these experiments.

Deletion Analysis Reveals Independent Promoter Activities Upstream Of Osx1 and Osx2—The basal activity of the Osx promoter was analyzed by making successive deletions from the 3' or 5'-end. These were inserted upstream of a promoterless pGL3 basic luciferase reporter. Fig. 4A shows a diagram of the −1269/+91 promoter with the locations of the Osx1 and Osx2 transcription start sites labeled for reference. Fig. 4B shows the effect of the deletions on promoter activity. The proximal promoter containing the Osx1, but not Osx2, start site had 40% of the activity of the full-length reporter (Fig. 4B, construct C versus A). Interestingly, deletion of the regions proximal to the Osx1 start site retained substantial activity (Fig. 4B, constructs D, E, and F versus A). Activity was lost with deletions upstream of −469 (Fig. 4B, constructs G, J, and K versus A). To determine whether there were independent promoter activities associated with the Osx1 and Osx2 start sites, −669/−469 and −269/+91 fragments were cloned upstream of the promoterless pGL3 basic. These reporters retained independent transcriptional activity that was 40% of the full-length −1269/+91 (Fig. 4B, constructs H and I versus A) and five times that of the pGL3 basic control (H versus L). As previously noted, the region around Osx1 was also capable of independent promoter activity (Fig. 4B, construct C versus A).

TNF Regulation of the Osx Promoter—The effect of TNF on the Osx promoter was then measured. Time course and dose response effects of TNF on the −1269/+91 Osx promoter were done in MC3T3 cells (Fig. 5, A and B) and also the primitive mesenchymal cell line C3H10T1/2 (Fig. 5, C and D). TNF caused a time- and dose-dependent inhibition of Osx promoter activity with an IC_{50} between 0.5 and 1.0 ng/ml consistent with the effects on Osx mRNA and the inhibition of OB differentiation.

Two major signal pathways mediating TNF action are NFκB and MAPK (11). The effect of NFκB expression on the promoter activity was determined by transfection of C3H10T1/2 cells with NFκB or its individual p50 or p65 subunits. Expression was driven with a pef-Myc-nuc vector containing an independent nuclear localization signal and driven by a cytomegalovirus promoter (18). Fig. 6 shows the results for this transfection and also the effect of other stimuli for comparison, including Runx2, Msx2, dexamethasone, 1,25(OH)_{2}D_{3}, or parathyroid hormone. Surprisingly, NFκB caused a potent stimulation of the Osx promoter. This effect was mediated by the p65 subunit of NFκB, which retained 40% of the activity of the intact dimer. The p50 subunit did not significantly increase Osx promoter activity. Msx2 expression increased Osx promoter activity 5-7-fold, but none of the other stimuli were effective. In a separate experiment, treatment with bone morphogenetic protein-2 (BMP-2) did not stimulate Osx promoter activity (not shown).

Deletions of the promoter were studied to localize the regions conferring the inhibitory effect of TNF. Fig. 7 shows the effect of TNF as fold stimulation relative to the activity of the respective control constructs. Most of the TNF inhibition was localized to a region between −514 to −510, as seen by the effect of deletion of this region (Fig. 7, constructs F and G versus A). This region was upstream of the Osx2 start site and embedded within the region of independent promoter activity shown in Fig. 4B. To further localize the TNF response element, small deletions or a four-base mutation were made within the −665/+91 construct (Fig. 7, constructs D and E). These localized TNF inhibition of the promoter to a region between −514 and −510. Confirmation of the localization was done by inserting three copies of the −520/−500 sequence upstream of the heterologous SV40 promoter (Fig. 7, construct H). TNF inhibited the activity of this promoter but had no activity on pSV40 alone (not shown).

Identification of Protein-DNA Binding in the TNF-responsive Region—EMSA was done using overlapping probes that spanned 200 bp around the TNF-responsive region to determine sites of nuclear
protein-DNA binding. Nuclear extract obtained from control and TNF-treated C3H10T1/2 cells was used for incubation with the probes. Five sites were found with strong protein-DNA interaction, one of which overlapped the TNF-responsive region (Fig. 8, probe 9, specific). Binding to probes 3 and 5 represented binding to the Myf5 consensus, whereas probe 6 spanned the Osx2 start site. Localization of the TNF response by deletion and mutational analysis led us to focus on a region within probe 9 (−520/−500); however, TNF did not change the pattern of binding at this site, although a small 2-fold increase in binding intensity was confirmed in repeated experiments. Incubation with antibodies to transcription factors was done using probe 9, but no supershifts were observed for p65, p50, RUNX2, RUNX1, JunD, RBP-Jκ, Fra-1, or Fra-2 (not shown).

**FIGURE 7.** Localization of TNF response regions in the Osx promoter. C3H10T1/2 cells were transfected with the promoter constructs shown (A–J) and the effect of TNF (10 ng/ml) on promoter activity was measured after 18 h. Promoter activity is shown at the right as fold change from the basal activity of that construct. In A, the location of the Osx1 and Osx2 start sites is shown for reference. Stippled vertical bars indicate the TNF-responsive region deduced from the results. LUC, luciferase.

**FIGURE 6.** NFκB stimulates the Osx promoter. The graph shows the activity of the −1269/+91 Osx promoter in C3H10T1/2 cells after treatment with various stimuli. Cells were simultaneously transiently transfected with the promoter reporter and an expression vector for NFκB (p50 + p65 subunits) or the individual NFκB subunits (p65 or p50). Results are also shown for the effect of RUNX2 or Msx2 expression or treatment with parathyroid hormone (PTH), dexamethasone (DEX), or 1,25(OH)2D3 (D). Mean ± S.E., * p < 0.05 versus control by ANOVA.

**FIGURE 5.** TNF inhibits the Osx promoter. Cells were transiently transfected with the −1269/+91 Osx promoter reporter and treated with TNF (10 ng/ml) 18 h later. A, time course of TNF inhibitory effect in MC3T3 cells. B, dose response of TNF in MC3T3 cells measured at 24 h of treatment. C, time course of TNF effect in C3H10T1/2 cells. D, dose response of TNF in C3H10T1/2 cells. * p < 0.05 versus time 0 (A and C) or versus 0 ng/ml (B and D) by ANOVA. Error bars denote mean ± S.E.

**FIGURE 4.** TNF Inhibition of Osx Is Mediated by MAPK Signaling—The TNF-bound p55 receptor activates a MAPK cascade in addition to the NFκB pathway. These pathways diverge downstream of activation of the cytosolic adapter protein TRAF2 in osteoblastic cells (11). We evaluated MAPK as a possible mediator of TNF inhibitory action, because NFκB paradoxically stimulated Osx transcription. Cells were pre-incubated for 2 h with PD98059, SP600125, or SB203580, inhibitors of MEK1 (the immediate upstream activator of ERK1/2), c-Jun-N-terminal kinase (JNK), or p38 kinase, respectively. The effect of these treatments on TNF inhibition of the Osx promoter was measured in the −1269/+91 Osx reporter. Fig. 9A shows that the MEK inhibitor reversed TNF inhibition of Osx transcription, whereas the inhibitors of JNK and p38 had no effect. The MEK inhibitor alone increased Osx promoter activity above the level of control. The effect of the MEK inhibitor was also observed using a reporter limited to the −669/−469 TNF-responsive region. The MEK inhibitor (PD98059) completely abrogated TNF inhibition of
this limited promoter and also increased basal activity (Fig. 9B). Fig. 9C shows that the MEK inhibitor also abrogated TNF inhibition of the heterologous SV40-LUC promoter bearing the 3′-H11002/520/-H11002500 sequence, indicating that the TNF element was sufficient to confer MAPK responsiveness. To determine whether the MEK inhibitor blocked TNF inhibition of Osx mRNA, MC3T3 cells were treated with PD98059 for 5 h before the addition of TNF. RNA was harvested 18 h later for measurement of Osx mRNA by real-time RT-PCR. Fig. 9D shows that the MEK inhibitor completely abrogated TNF inhibition of Osx mRNA. PD98059 alone caused a significant increase in steady state Osx mRNA. Similar results were obtained for the -1269/+91 Osx promoter using a dominant negative MEK1 or ERK1. Fig. 10 shows that these dominant negatives partially reversed TNF inhibition of the promoter, confirming the results obtained using PD98059.

Identification of the NFκB Regulatory Element—The pattern of NFκB stimulation of the promoter was determined by overexpression of the p65 subunit, as described for Fig. 6. NFκB responsiveness was abolished with deletion of the proximal promoter between -1269/-91 Osx promoter reporter, and activity was measured 18 h later. PD98059 partially abrogated TNF inhibition and increased basal promoter activity. B, as described for A but using a reporter restricted to -469/-669 spanning the TNF-responsive region. C, as described for A using three copies of the -514/-500 TNF-responsive sequence upstream of a minimal SV40 promoter. D, TNF inhibition of Osx mRNA is prevented by pretreatment with a MEK1 inhibitor. C3H10T1/2 cells were treated with PD98059 5 h prior to the addition of TNF (10 ng/ml), and RNA was isolated 18 h later. Osx mRNA was measured by real-time RT-PCR. Mean ± S.E. Bars labeled with different letters (a, b, and c) are significantly different from each other (p < 0.05 by ANOVA).
The sequence bound was concordant with the functional sequence identified by deletion and mutational analysis of the promoter. This sequence is compared with the NFkB consensus in Fig. 12C. Binding of recombinant p50 to this sequence was of very low affinity compared with binding to the NFkB consensus sequence (Fig. 12D). Nuclear protein from TNF-treated C3H10T1/2 cells did not bind this low affinity site, demonstrating that the amount of NFkB stimulated by TNF was insufficient to bind and activate the enhancer (Fig. 12E, lane 5).

To confirm that NFkB activation was not modulating TNF inhibition of the Osx promoter, NFkB activation was blocked by siRNA-p65 or expression of a degradation-resistant N terminus-deleted IkB (ΔIkB, (19)) in C3H10T1/2 cells. Fig. 13A shows that neither the siRNA-p65 nor the ΔIkB could prevent TNF inhibition of Osx promoter activity. Fig. 13B shows the efficacy of siRNA-p65 in blocking TNF stimulation of an NFkB-dependent reporter.

**DISCUSSION**

Our results show that TNF regulates expression of Osx by inhibiting the transcriptional activity of its promoter. The low dose of TNF that inhibits Osx expression is similar to doses that inhibit the differentiation of OB (6, 14). TNF inhibition of Osx was dose- and time-dependent and observed in two cell lines representative of the early stages of OB differentiation. We have previously shown (12) that TNF inhibits the expression of RUNX2, another critical transcription factor required for OB differentiation (12). Previous studies have shown that both RUNX2 and Osx are needed for OB differentiation. Thus, the effect of TNF can be attributed to suppression of both factors during the early events in OB differentiation.

TNF action on Osx is likely to be transcriptional. First, there was no effect of TNF on Osx mRNA stability. Second, treatment with cycloheximide, although inhibitory on its own, was unable to prevent a further inhibitory action of TNF. These data suggest that the effects of TNF are direct rather than requiring the indirect induction of a protein mediator. The requirement for protein synthesis has been suggested for the stimulation of Osx mRNA by BMP, which is prevented by cycloheximide treatment (20).

To evaluate TNF action on the Osx promoter, we cloned a 1269-bp fragment upstream of the luciferase reporter. 5′-RACE and primer extension analysis revealed two transcription start sites. One of these, termed Osx1, represented the start site for the more abundantly expressed and previously described mRNA species. Previous work (16, 17) has shown that the human Osx mRNA has at least two isoforms. These include an abundant form highly homologous to the murine Osx and a scarcer alternatively spliced form. Using selective primers, we confirmed the expression of the two isoforms in MC3T3 cells and hypothesized that they arose from the two transcription start sites. Our data also show that the Osx promoter contains at least two regions capable of independent promoter function. This suggests that the two mRNA isoforms could be expressed under the regulation of the two different promoters, although full transcriptional activity requires both. Osx2 mRNA could function similarly to Osx1, have a unique function, or be expressed as an unstable species that is rapidly removed. Further work will be needed to determine the function of Osx2, its regulation, and whether two protein forms are translated. Osx1 appears to be the major form of Osx mRNA.

TNF inhibited the activity of the Osx promoter in a dose- and time-
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FIGURE 12. Analysis of NFκB or nuclear protein binding to the NFκB response region of the Osx promoter. A, map showing the NFκB-responsive region determined in deletion and mutation studies and the sequences of probes 1–5 used in the EMSA. B, binding of recombinant p65, p50, or the p65/p50 heterodimer to the probes shown in A. p50 monomer and the p50/p65 heterodimer bind to probes 2 and 4 with some binding to probe 3. C, comparison of the core binding sequence localized by EMSA and also through deletion and mutational analysis to a consensus NFκB binding sequence. D, EMSA comparing binding of NFκB to the consensus (left) and Osx promoter element (right) shows the Osx element binding to be of low affinity. E, EMSA using probe 2 with nuclear extract from C3H10T1/2 cells treated with control or TNF (10 ng/ml). TNF does not induce sufficient NFκB to bind the Osx element (lane 5) compared with the recombinant p50 (lane 1). c, empty vector control for NFκB subunits (lane 2) or vehicle control for TNF (lane 4).

Dependent manner in both C3H10T1/2 and MC3T3 cells. These results were similar to the dose- and time-dependent regulation of Osx mRNA, with a small delay in TNF action that can be attributed to the longer half-life of luciferase mRNA. Deletion analysis of the promoter localized the inhibitory effect of TNF to −514/−510, a region proximal to the Osx2 start site. This region and its flanking sequence contain consensus sites for thyroid transcription factor (TTF1), pleomorphic adenoma gene 1 (PLAG1), AP-2, and the RBP-Jκ site. Although TNF did not change the pattern of binding at this site, a small 2-fold increase in binding intensity was observed. Regulation at this site could occur through phosphorylation of one of the proteins in the bound complex, thereby changing the regulatory effect of the complex from an enhancer to a suppressor. Further investigation will be needed to identify the protein in this complex that is regulated by TNF.

TNF signals through multiple intracellular pathways (11). We designed experiments to distinguish the roles of two major TNF pathways, MAPK and NFκB, as mediators of TNF inhibition of Osx transcription. In osteoblasts, a TNF trimer binds two receptor forms, TNFSF1R or TNFSFR2, of which only the TNFSF1R (type 1, p55) mediates inhibition of OB differentiation (14). In a well-established paradigm, the bound receptor activates a large cytosolic complex that includes TRAF2 and IkB kinase isozymes. The IkB kinase isozyme then phosphorylates IkB, an NFκB binding protein that normally sequesters NFκB in the cytoplasm. Phosphorylation of the IkB N terminus leads to its degradation and liberation of NFκB for nuclear entry and gene regulation. However, TRAF2 also stimulates the MAPK cascade with downstream activation of ERK1/2, p38, or JNK. Our data suggest that TNF inhibition of Osx expression is mediated via MAPK, because the MEK1 inhibitor PD98059 prevents TNF effects on Osx promoter activity and Osx mRNA expression. This blockade of TNF action was limited to the inhibitor of MEK and not observed with inhibitors of p38 or JNK. The MEK inhibitor alone stimulated basal Osx promoter activity and mRNA expression, suggesting that a tonic inhibitory influence of MAPK on the Osx promoter must be active. The MEK inhibitor PD98059 also abrogated TNF inhibition of a reporter containing the isolated −669/−460 region or the −520/−500 core response element cloned upstream of a heterologous promoter. Thus, our results on localization of TNF action and mediation of the TNF signal by MAPK are concordant. Although our results exclude a role for JNK or p38 in TNF regulation of Osx, PD98059 could still inhibit additional kinases. Data supporting MEK/ERK as the responsible pathway for TNF action was also supported by the partial abrogation of TNF action by dominant negatives MEK1 and ERK1. Further work will be needed to define the MAPK utilized by TNF and the nuclear protein target of this phosphorylation cascade.

Previous studies provide conflicting evidence on the role of MAPK in OB differentiation that may be explained by specific actions of the individual pathways. Commitment of pluripotent precursors to the OB phenotype is stimulated by a variety of factors, including BMPs that increase expression of the key transcription factor RUNX2 or that modify Osx expression. The sensitivity to such stimuli may be increased or decreased by the different MAPK pathways. Stimulation of OB differentiation by BMP-2 has been shown to require p38 kinase (21–24). MEK blockade, which would block activation of ERK1/2, is synergistic with BMP-2 action, consistent with this idea. In support of this concept,
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Higuchi et al. (32) establish that chronic suppression of MEK increases markers of differentiation, including expression of alkaline phosphatase and osteocalcin in MC3T3-E1 cells and of alkaline phosphatase in more primitive pluripotent C2C12 cells. Recently, Osyczka and Lebov (25) showed that inhibition of MEK using PD98059 permitted BMP stimulation of OB phenotypic gene expression in human marrow stromal cells, whereas a constitutively active MEK inhibited this response. In parallel with our findings on TNF, ERK blockade has been shown to prevent inhibition of OB differentiation by other factors, including EGF and FGF (26). These results suggest that some inflammatory signals via ERK inhibit OB differentiation, whereas others via p38 stimulate OB differentiation.

There are clear exceptions to an inhibitory effect of ERK. Cell contact with matrix is one signal that activates ERK and phosphor-ylates RUNX2, an obligatory step for RUNX2 organization of skeletal gene expression (27–30). More recently, prevention of pre-osteoblast apoptosis by wingless factors (wnt) was shown to be ERK-dependent (31). Thus, ERK effects may depend on whether the stimulus also activates other signal pathways or on the stage of cell differentiation (32). Finally, although the MAPK inhibitors used in the present study distinguish between ERK, p38, and JNK, there may still be other closely related kinases to MEK/ERK that could be inhibited by PD98059 that will have to be evaluated (33).

Because TNF also stimulates activation of NFκB, we evaluated the effect of NFκB expression on the Osx promoter. Our results indicate that NFκB does not mediate the inhibitory effect of TNF, as blockade of NFκB activation did not prevent TNF action. Surprisingly, targeted expression of NFκB to the nucleus using a strong cytomegalovirus promoter containing an independent nuclear localization signal unmasked a potent enhancer function in the proximal Osx promoter. This enhancer was not activated by TNF, as TNF does not stimulate a sufficient elevation of nuclear NFκB to bind the low affinity site. Nevertheless, the potency of transcriptional activation observed using the NFκB expression vector suggests that the enhancer might be an important regulator of Osx expression and OB differentiation. Such an enhancer might be functional under other circumstances that increase higher levels of NFκB. NFκB is activated by a variety of stimuli other than TNF, including members of the TNF superfamily that have not been studied for effects on OB (34). In addition, the potency of NFκB to stimulate Osx transcription could be modulated by phosphorylation of p65 on key serines and tyrosines shown to modulate p65 action at other targets (18). Such a modulation of NFκB potency at its enhancer could serve to balance the negative inflammatory stimulus of TNF via MAPK. Additional information is needed to determine the role of NFκB in Osx expression and OB differentiation.

Bone remodeling is a coupled process that balances the rate of resorption with formation. Continued recruitment of new OB from the precursor pool must occur to counteract the resorption stimulus associated with estrogen deficiency at menopause or in inflammatory arthritis. TNF impairs the recruitment of OB and thus blunts the magnitude of bone formation to further shift skeletal balance toward a catabolic state. The inhibition of Osx by TNF may contribute to the mechanism of suppressed OB differentiation.

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