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Inhibition of Osteoblast Differentiation by Tumor Necrosis Factor-α*

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ABSTRACT

Tumor necrosis factor-α (TNF-α) has a key role in skeletal disease in which it promotes reduced bone formation by mature osteoblasts and increased osteoclastic resorption. Here we show that TNF inhibits differentiation of osteoblasts from precursor cells. TNF-α treatment of fetal calvaria precursor cells, which spontaneously differentiate to the osteoblast phenotype over 21 days, inhibited differentiation as shown by reduced formation of multilayered, mineralizing nodules and decreased secretion of the skeletal-specific matrix protein osteocalcin. The effect of TNF was dose dependent with an IC_{50} of 0.6 ng/ml, indicating a high sensitivity of these precursor cells. Addition of TNF-α from days 2–21, 2–14, 7–14, and 7–10 inhibited nodule formation but addition of TNF after day 14 had no effect. Partial inhibition of differentiation was observed with addition of TNF on only days 7–8, suggesting that TNF could act during a critical period of phenotype selection. Growth of cells on collagen-coated plates did not prevent TNF inhibition of differentiation, suggesting that inhibition of collagen deposition into matrix by proliferating cells could not, alone, explain the effect of TNF. Northern analysis revealed that TNF inhibited the expression of insulin-like growth factor I (IGF-I). TNF had no effect on expression of the osteogenic bone morphogenic proteins (BMPs-2, -4, and -6), or skeletal LIM protein (LMP-1), as determined by semiquantitative RT-PCR. Addition of IGF-I or BMP-6 to fetal calvaria precursor cell cultures enhanced differentiation but could not overcome TNF inhibition, suggesting that TNF acted downstream of these proteins in the differentiation pathway. The clonal osteoblastic cell line, MC3T3-E1–14, which acquires the osteoblast phenotype spontaneously in postconfluent culture, was also studied. TNF inhibited differentiation of MC3T3-E1–14 cells as shown by failure of mineralized matrix formation in the presence of calcium and phosphate. TNF was not cytotoxic to either cell type as shown by continued attachment and metabolism in culture, trypan blue exclusion, and Alamar Blue cytotoxicity assay. These results demonstrate that TNF-α is a potent inhibitor of osteoblast differentiation and suggest that TNF acts distal to IGF-I, BMPs, and LMP-1 in the progression toward the osteoblast phenotype. (*Endocrinology* **141:** 3956–3964, 2000)

TUMOR NECROSIS FACTOR (TNF)-α is one of several cytokines produced in excess in postmenopausal osteoporosis and within the joint space in rheumatoid arthritis (1–4). TNF-α has been shown to reduce bone formation by inhibiting the production of matrix proteins by phenotypically mature osteoblasts and to promote osteoclastic resorption. The loss of bone after estrogen withdrawal can be abrogated by sequestration of TNF with soluble TNF receptors, revealing a key role for TNF in postmenopausal osteoporosis (5, 6). Work from several laboratories has revealed an inhibitory effect of TNF-α on the synthesis of type I collagen and induction of osteoblast resistance to vitamin D, as shown by inhibition of 1,25-dihydroxyvitamin D_3 stimulated production of osteocalcin (7–14). These actions of TNF shift the formation/resorption balance in the skeleton toward resorption, which leads to fractures in postmenopausal osteoporosis and periarticular bone loss in inflammatory arthritis. Although the suppressive effects of TNF-α on the function of mature osteoblasts has been described, little is known about the effects of TNF-α on the differentiation of osteoblasts from their precursor cell pool. We considered that TNF might also inhibit the recruitment of osteoblasts from their stromal progenitor cells.

Osteoblasts derive from a pool of pluripotent stem cells capable of differentiating toward a number of phenotypes (15–18). Stem cells that are destined to become osteoblasts must achieve an osteoblastic trajectory rather than proceed along an adipocytic, myocytic, or fibroblastic path. A number of secreted and intracellular mediators have been suggested to promote the differentiation and survival of osteoblasts. These factors promote a succession of cellular events that include precursor cell proliferation, growth arrest, phenotype selection, and finally, osteoblast-specific gene expression (19). Paracrine factors suggested to support osteoblast differentiation include bone morphogenic proteins-2, -4, and -6, and IGF-I as well as nuclear protein transcription factors (20–30). TNF-α could potentially regulate any of these cellular events in the differentiation pathway. In addition, TNF has been suggested to regulate apoptosis of osteoblasts, a mechanism that could accelerate the exit of osteoblasts or their precursors from their functional pool (31–35).

We studied the effect of TNF-α on spontaneous differentiation of precursor cells toward the osteoblast phenotype and on enhancement of differentiation by IGF-I and BMP-2. To do this, we used two models of osteoblast differentiation, fetal rat calvaria preosteoblasts and a murine calvaria clonal osteoblastic cell line, MC3T3-E1–14. Fetal calvaria cells acquire the osteoblast phenotype in postconfluent culture in

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the presence of ascorbate. Over a 3-week period, precursor cells grow to confluence and form multilayered nodules that mineralize and secrete the osteoblast-specific matrix protein, osteocalcin. The clonal MC3T3-E1–14 cells also spontaneously differentiate under these conditions and secrete a matrix competent for mineralization. Here we show that TNF-α is a potent suppressor of osteoblast differentiation in these experimental models at the point of phenotype selection in the differentiation pathway.

Materials and Methods

Reagents

Reagents were obtained from the following sources: Human TNF-α and IGF-I were purchased from PeproTech, Inc. (Rocky Hill, NJ), human BMPs-2, -4, and -6 were generous gifts from Genetics Institute, Inc. (Cambridge, MA), human PTH (1–32) from Peninsula Laboratories, Inc. (Belmont, CA), Types I and II collagenase from Worthington Biochemical Corp. (Lakewood, NJ), and Earle's minimum essential medium (MEM) from Life Technologies, Inc. (Grand Island, NY). Heat-inactivated FBS was purchased from HyClone Laboratories, Inc. (Logan, UT), Dulbecco's PBS (without calcium and magnesium), trypsin/Versene, sodium bicarbonate solution, HEPES, and penicillin/streptomycin were purchased from BioWhittaker, Inc. (Walkersville, MD). BGJb (Fitton-Jackson modification) was from either Life Technologies, Inc. (liquid medium) or Sigma (powdered medium, St. Louis, MO). Other cell culture reagents were purchased from Sigma. Cell culture plates coated with rat tail collagen I were purchased from Becton Dickinson and Co. (Bedford, MA), and TRIzol Reagent from Life Technologies, Inc. Actinomycin-D-Mannitol was purchased from Sigma and Alamar Blue from BioSource International, Inc. (Camarillo, CA). Primers for RT-PCR and probes for Northern analysis were synthesized by the Emory University Microchemical Facility (Atlanta, GA). Dr. Lawrence Phillips (Emory University, Atlanta, GA) kindly provided a full-length IGF-I complementary DNA (cDNA). Zeta-Probe GT Genomic Tested Blotting Membranes were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA) and ^{32}P-dCTP was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The probe for GAPDH was prepared by RT-PCR. GeneAmp RNA PCR Core Kits were purchased from PE Biosystems (Foster City, CA).

Fetal rat calvaria cultures

The Emory University and VA Medical Center animal use committee approved all procedures. Timed pregnant Sprague Dawley rats were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Cultures of primary and secondary fetal rat calvaria cells were prepared as previously described with the exception that the primary culture of digested fetal calvaria cells was allowed to incubate for 8 days rather than 7 (3 days postconfluent) (36). Briefly, frontal and parietal bones were dissected from day 22 fetal rat calvaria and subjected to four sequential, 20 min digestions with a mixture of Types I and II collagenase. Cells in fractions three and four were washed, combined, and cultured at 0.8–1.0 $\times$ 10^6 cells per 75 cm^2 flask in Earle's MEM + 10% FBS. After 8 days, during which the medium was replaced twice, the primary cells were subcultured using trypsin/Versene and plated at 10^5 cells/2 ml per well in 6-well plates. These secondary cultures were grown in MEM + 10% FBS until confluent (7 days), then switched to MEM + 10% FBS + 50 μg/ml L-ascorbic acid for the next 7 days. On day 14 after plating, the medium was switched to BGJb (Fitton-Jackson modification) + 10% FBS + 5 mM β-glycerophosphate for the final 7 days of culture. The medium was changed on days 4, 11, and 18.

Mineralized nodules were fixed with 70% ethanol on day 21, stained with the von Kossa technique (37), and counted using an Optomax V HR image analyzer (Hollis, NH). Total nodule number, including nonmineralized nodules, was assessed by counting each culture 5 times after staining with hematoxylin. The addition of TNF-α, BMP-6, or IGF-I to the culture medium is described for each experiment. The assessment of TNF inhibition of unmineralized and mineralized nodule formation was done relative to control cultures grown in the absence of TNF.

MC3T3-E1–14 clonal osteoblastic cultures

The clonal osteoblastic cell line, MC3T3-E1, clone 14, was kindly provided by Dr. Rene Franceschi (University of Michigan, Ann Arbor, MI). Stock cultures were grown in MEM + 10% FBS. For experiments, cells were plated in MEM + 10% FBS (1.9×10^5 cells/ml/well in 12-well plates) and switched to α-MEM + 10% FBS + 50 μg L-ascorate the next day. Mineralization was induced by adding 10 mM β-glycerophosphate to this medium on day 8 after plating. Because these cells are clonal, differentiation occurs throughout the culture, unlike the fetal calvaria cells that form discrete nodules. Von Kossa staining was done as indicated on day 16 of culture, by which time control cells were uniformly mineralized.

Osteocalcin assay

Culture supernatants were collected and stored at −70 C until assayed for osteocalcin levels by the Biomedical Technologies competitive rat osteocalcin RIA (Stoughton, MA).

Cytotoxicity assay

The cytotoxicity assay was adapted from the method of Ahmed *et al.* (38). Serial dilutions of TNF-α and Actinomycin D were prepared in MEM + 10% FBS in duplicate rows of 96-well plates (100 μl/well). A suspension of MC3T3-E1–14 cells (10^4 cells/100 μl/well in MEM + 10% FBS + 200 U/ml penicillin + 200 μg/ml streptomycin) was added to the plates. The 96-well plates were incubated for 4 days in a humidified incubator at 37 C with 5% CO_2. Alamar Blue (40 μl/well, diluted 1:2 in MEM + 10% FBS) was added and the plates were incubated for an additional 6 h. Cell growth was measured as the absorbance at 570 nm minus the absorbance at 620 nm using a 96-well plate reader (Bio-Tek Instruments, Inc., Model EL311).

Northern analysis and RT-PCR

Total cellular RNA was prepared from fetal rat calvaria cultures by adding TRIzol (1 ml per well of a 6-well plate) to lyse the cells. Chloroform was added (0.2 ml/sample) to separate the aqueous and organic phases, followed by precipitation of the RNA from the aqueous phase with isopropanol (0.5 ml per sample). Northern analysis for IGF-I was carried out by fractionating total RNA in a 2.2 M formaldehyde gel followed by capillary transfer to Zeta-Probe GT Genomic Tested Blotting Membrane. IGF-I messenger RNA (mRNA) species were detected using a full-length rat IGF-I cDNA after random primer labeling with ^{32}P-dCTP. Membranes were stripped and rehybridized with a human GAPDH cDNA probe. mRNA band intensity was quantitated using a Molecular Dynamics, Inc. phosphorimager (Sunnyvale, CA) and results were calculated as IGF-I/GAPDH. Semiquantitative RT-PCR was carried out using 0.5 μg total cellular RNA per reaction. Preliminary experiments showed that 22 cycles were well within the linear range of

TABLE 1. Primer sequences used for semi-quantitative RT-PCR analysis of mRNA levels of osteogenic proteins

Gene	Forward primer sequence (5′ → 3′)	Reverse primer sequence (5 → 3′)
BMP-2	CACAAGTCAGTGGGAGAGC	GCTTCCGCTGTTTGTGTTTG
BMP-6	CAGCTTGCAAGAAGCATGAG	GGAAGTAAAGAACCGAGATG
LMP-1	ATCCTTGCTCACCTCACGGG	GCACTGTGCTGGTTTTGTCTGG
18S rRNA	ACCTGGTTGATCCTGCCAGT	GATAGGGCAGACGTTCGAAT

Northern analysis was used for IGF-I.

amplification for each gene being measured. The primers used are shown in Table 1. Primers were end labeled with ^{32}P-[ATP-γ] using T_4 kinase (39). Results were quantitated using a phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA) and corrected for 18S RNA amplified from the same samples in the PCR reaction.

Statistics

ANOVA is used to determine statistical differences between groups. Multiple comparisons between individual groups are assessed by the method of Tukey. In legends, P = NS indicates $P \geq 0.05$.

Results

During the initial 7 days of culture, secondary fetal rat calvaria cells proliferate to form a confluent monolayer with a uniform appearance. After the addition of L-ascorbic acid to the medium on day 7, progression to the osteoblast phenotype begins and osteoblastic nodules begin to form by day 14. Addition of phosphate to the medium, as β-glycerophosphate, during the third week of culture promotes the deposition of calcium phosphate into nodules, which can be stained with von Kossa reagent and counted by computerized image analysis. Figure 1A shows that addition of TNF-α to fetal rat calvaria preosteoblasts dramatically reduces the number of multilayered, mineralized nodules. Control cultures show typical nodules and internodular areas filled with a cobblestone pattern of confluent cells. After TNF-α treatment, however, nodules fail to appear, but the remaining confluent cells appear intact. TNF-α treated cells remain attached to the culture plate. Trypan blue staining of control and TNF-α-treated cultures shows no uptake of stain, confirming viability. To determine the time course of sensitivity to TNF-α by the differentiating cultures, TNF-α (100 ng/ml) was added and maintained during days 2–21, 7–21, or 14–21. Figure 1B shows that addition of TNF-α day 7–14 is sufficient to produce maximal inhibition at the 100 ng/ml dose. Control cultures and cultures treated with TNF-α (100 ng/ml) beginning on day 14 show no significant difference in the number of total nodules by day 21. The apparent increase in nodules after treatment with TNF days 14–21, seen in Fig. 1C, was not observed consistently (total nodules observed in repeat experiment, TNF 10 ng/ml days 14–21: control 100 ± 5%, TNF 107 ± 1.1%, n = 3 wells/group, $P > 0.05$ by Student's t test). Nodule counts were similar on day 17, indicating no loss of nodules once they were formed (day 17 counts of total nodules: control 329 ± 8.5, TNF, 340 ± 7.7; day 21 counts of total nodules: control 315 ± 10.7, TNF 316 ± 5.8 nodules/well; n = 6/group, $P > 0.05$). These results suggest that TNF-α inhibits entry of cells into the differentiation pathway but does not cause loss of osteoblastic nodules once they are formed. Treatment of cultures with TNF-α inhibited mineralized as well as total nodules as shown in Fig. 1C.

To further determine the critical period of sensitivity to TNF-α, shorter exposures to TNF-α were used. Figure 2 shows that a 24-hour exposure between days 7 and 8 produces significant dose-dependent reduction in nodule numbers compared with those observed in control cultures. Additional dose-dependent reductions are observed if the exposure time is lengthened to 4 days (days 7–11) or 7 days (days 7–14). As in the previous experiment, no greater decrease in nodule formation is observed when cultures are

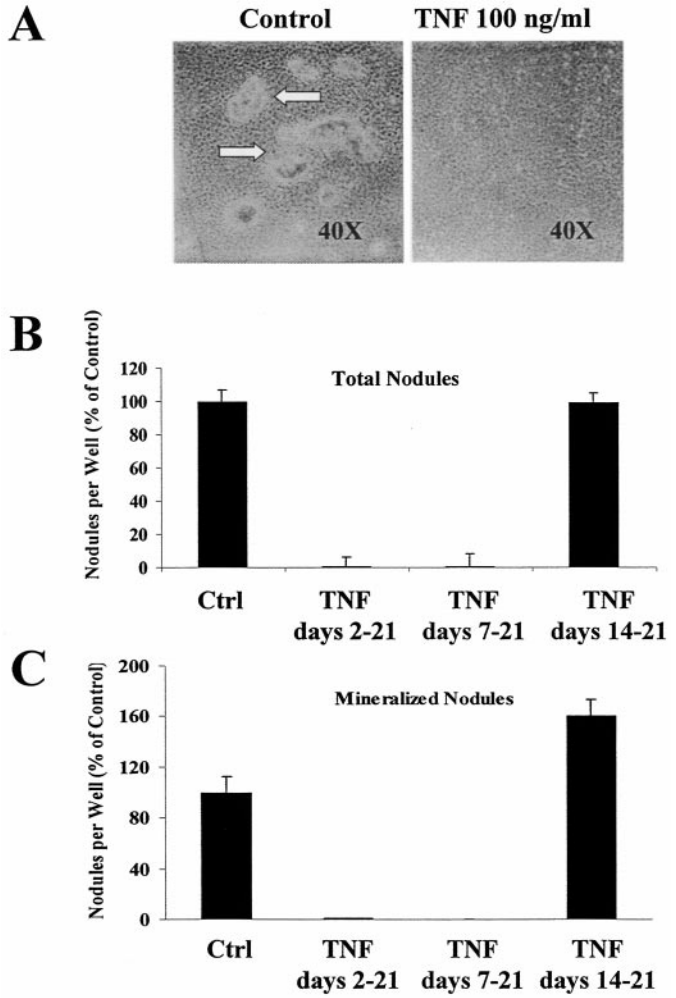

FIG. 1. TNF-α inhibits osteoblast differentiation. TNF-α (100 ng/ml) was added to fetal calvaria cultures beginning on days 2, 7, or 14 and maintained until day 21 as noted for each figure. Total and mineralized nodules were counted on day 21. A, Phase contrast microscopy of control culture showing nodule formation (*left photo*). TNF-α (100 ng/ml days 2–21) treated culture showing undifferentiated confluent precursor cells at day 21 (*right photo*). B, Effect of adding TNF-α (100 ng/ml) on days 2, 7, or 14 on total nodule formation (mineralized + unmineralized) as counted on day 21 of culture. Mineralized nodules account for 75% of total nodules on average. C, Effect of TNF-α on mineralized nodules. Conditions identical to B. Results shown are representative of 2–3 experiments, mean ± SEM, n = 3 wells/group. TNF groups (days 2–21 or 7–21) differ from Control and TNF days 14–21 by ANOVA, $P < 0.05$. Control and TNF days 14–21 are not different by ANOVA. Control, No TNF added.

treated past day 14 (days 7–21). Figure 3A (*solid line*) shows the dose response effect of TNF-α and reveals an IC_{50} of 0.6 ng/ml when TNF-α is present continuously during days 7–21. We also measured the osteoblast specific protein, osteocalcin, as an index of differentiation. The level of secreted osteocalcin, which is directly proportional to the number of nodules, is also dose-dependently inhibited by TNF-α (Fig. 3A, *dashed line*). The presence of TNF-α between days 7 and 21 also prevents full maturation of nodules as measured by a dose-dependent reduction in the percentage of nodules that undergo mineralization (Fig. 3B).

A competent matrix is necessary for the differentiation of

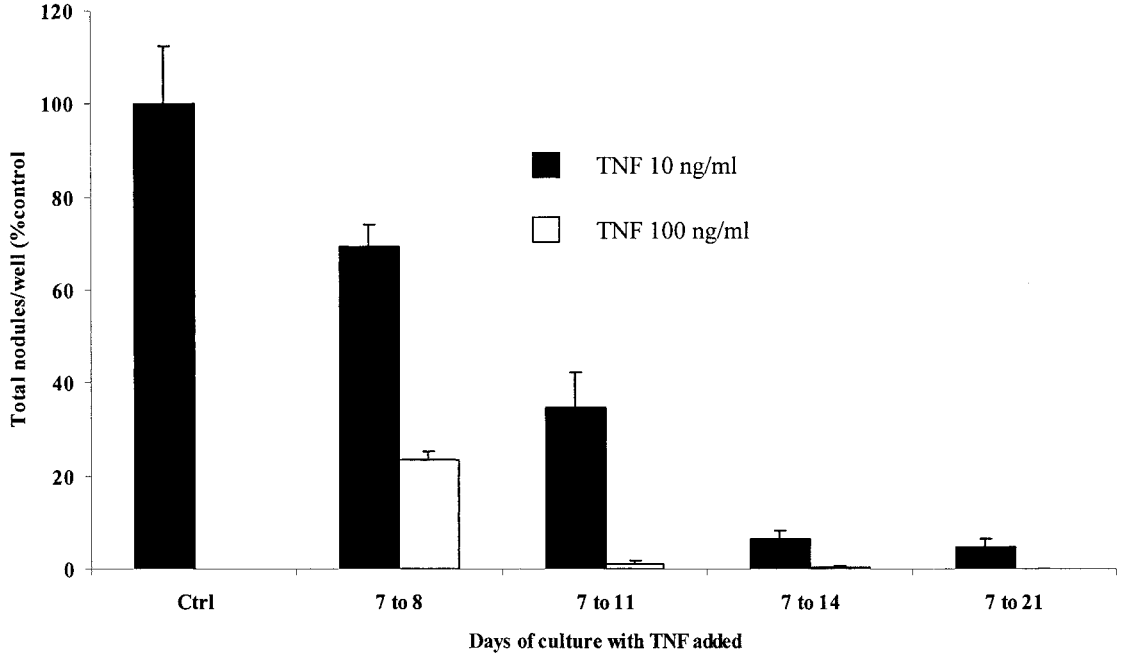

FIG. 2. Effect of abbreviated TNF-α treatment (10 or 100 ng/ml) on the number of total nodules counted on day 21. TNF-α was present on days 7–8, 7–11, 7–14, or 7–21 in fetal calvaria cultures. At the end of the indicated treatment period, TNF-α was removed by changing the medium. Results shown are representative of 2–3 experiments, mean ± SEM, n = 3–6 wells/group. All groups differ from Control by ANOVA, $P < 0.05$. Control, No TNF added.

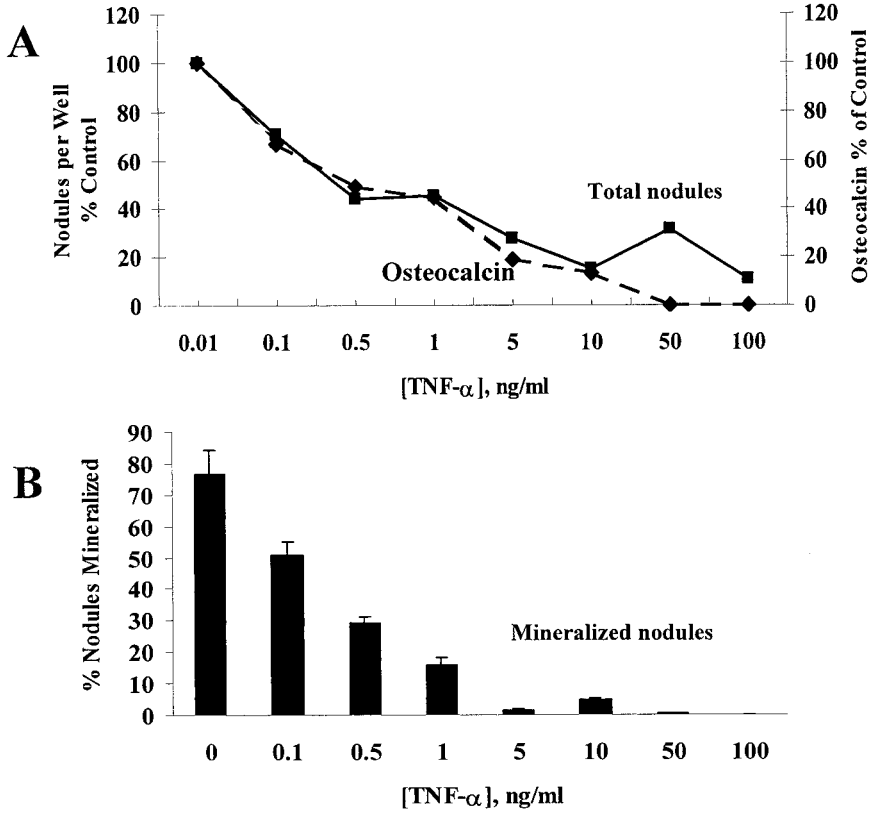

FIG. 3. Dose response inhibitory effect of TNF-α on total nodule formation, osteocalcin secretion, and mineralization. TNF-α was added on days 7, 10, 14, and 17 of fetal calvaria cultures. Cultures were fixed on day 21 and mineralized nodules were stained with von Kossa silver stain, followed by hematoxylin as described in *Materials and Methods*. Data are mean ± SEM of 5–6 cultures. A, Effect of TNF-α on total nodule numbers and osteocalcin levels. B, Effect of TNF-α on the percentage of total nodules mineralized. All groups differ from control (TNF 0 ng/ml) by ANOVA, $P < 0.05$.

osteoblasts from their precursors. Nodules do not form in culture in the absence of ascorbate because of inadequate collagen production (not shown) (40). TNF-α is known to inhibit the production of type I collagen, an essential constituent of this competent matrix (41). To determine if TNF-α inhibition of type I collagen synthesis is sufficient to explain suppression of differentiation, fetal calvaria cells were grown on rat tail collagen I-coated tissue culture plates and the effect

of TNF-α was determined. Figure 4 shows that osteoblasts grown on collagen-coated plates produce the same number of nodules as those grown on uncoated plates. Despite the presence of type I collagen on the coated plates, the addition of TNF-α from days 2–14 suppresses osteoblast differentiation and prevents nodule formation. Thus, TNF-α acts as an inhibitor even in the presence of a type I collagen matrix.

The effect of TNF-α was also studied in MC3T3-E1 cells, which differentiate in culture. We used the Clone 14 cell line (MC3T3-E1–14) that forms a mineralized matrix after 16 days in culture in the presence of ascorbate and β-glycerophosphate (42). Figure 5A shows that continuous exposure of these cells to TNF-α (0.01–10 ng/ml, days 1–16) completely prevents mineralization in a dose-dependent manner. Un-

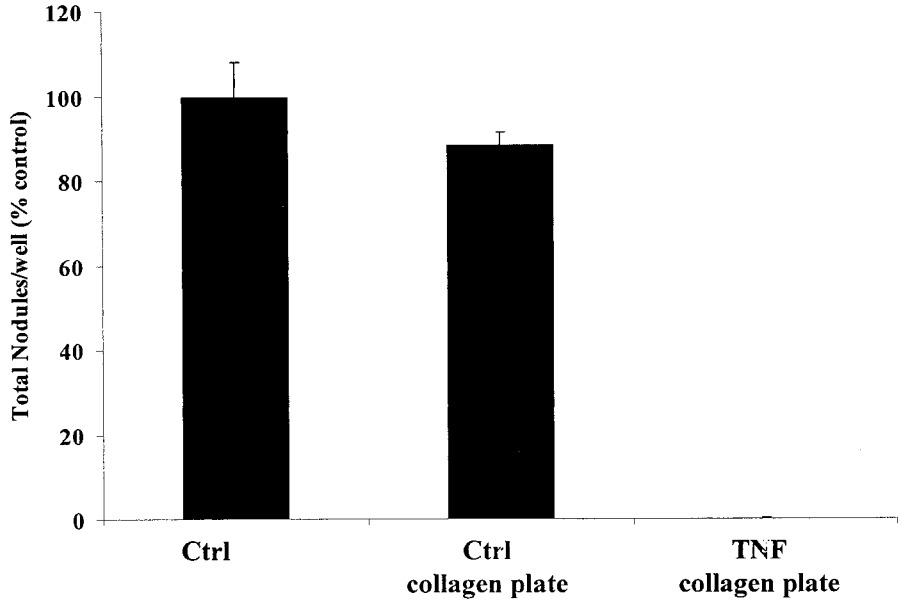

FIG. 4. Determination of the ability of fetal rat calvaria cultures to form nodules on collagen-coated plates. Cells for control cultures were plated on both regular tissue culture plates and plates coated with rat tail collagen I. Cells to be treated with TNF-α were plated on collagen-coated plates. TNF-α (10 ng/ml) was added to the cultures days 7–21. Total nodules were counted on day 21. n = 2–3 cultures, means ± SEM. TNF group differs from both Control groups by ANOVA, $P < 0.05$. Control, No TNF added for cultures grown with (bar 2) or without (bar 1) collagen coating on the plates.

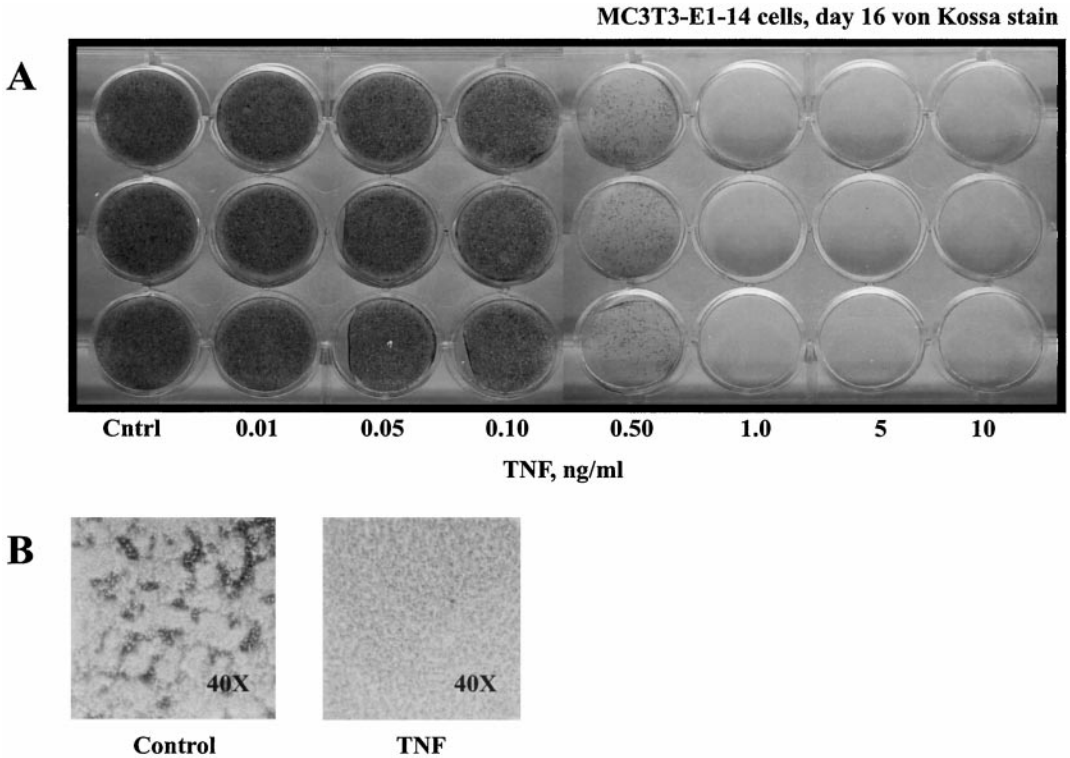

FIG. 5. A, TNF-α inhibits osteoblast differentiation of MC3T3-E1–14 cells in a dose-dependent manner. TNF-α (0.01–10 ng/ml) was added to cultures on days 1, 3, 6, 8, 10, and 13 and von Kossa staining was done day 16. Control = no TNF added. B, Phase contrast microscopy of MC3T3-E1–14 cultures showing diffusely mineralized control cells (*left photo*, no TNF) and unmineralized undifferentiated cells (*right photo*, TNF, 10 ng/ml).

differentiated cells remained attached and viable as shown in Fig. 5B. The inhibition of differentiation in MC3T3-E1–14 cells is not associated with increased cell death, as shown by cytotoxicity assay using the metabolic indicator dye, Alamar Blue. Figure 6 shows that doses of TNF-α up to 5000 ng/ml did not cause a reduction in cell viability compared with control cultures. Actinomycin D, a positive control, decreased viability as expected. Although this experiment was done after 4 days of TNF treatment, a repeat experiment done after 16 days of TNF (10 ng/ml) showed no cytotoxicity.

The protein products of several genes are known to promote osteoblast differentiation. We measured the steady-state levels of mRNA for insulin-like growth factor-I (IGF-I), bone morphogenic proteins (BMPs -2, -4, and -6), and skeletal LIM protein (LMP-1) to determine if TNF-α suppresses expression of these factors. Northern analysis was used to measure steady-state IGF-I mRNA species and semiquantitative RT-PCR was used to measure mRNA levels of BMP-2, -6, and LMP-1 in fetal rat calvaria cultures. Figure 7 shows a representative Northern analysis from day 7 fetal calvaria cell cultures treated with TNF (100 ng/ml). TNF potently decreases the steady-state level of IGF-I mRNA species as measured 16 h after addition of TNF (T) compared with control cultures (C) as previously reported (43). Table 2 quantitates the inhibition of IGF-I mRNA species as % GAPDH control. Table 2 also shows the effect of TNF-α on BMP steady-state mRNA. Semiquantitative RT-PCR does not reveal any effect of TNF on BMPs -2, -6, or LMP-1, shown as % control after correction for 18S ribosomal RNA (Table 2). To determine if suppression of IGF-I by TNF-α explains the inhibition of differentiation, cultures were treated with TNF-α, IGF-I, or both. IGF-I was added to calvaria cultures on day 7 and maintained until day 21. Figure 8 shows that IGF-I alone stimulates nodule formation well above the numbers produced by control cultures. Despite the positive effect of IGF-I on differentiation, TNF-α (100 ng/ml) is still able to suppress differentiation in IGF-I treated cultures. Submaximal concentrations of TNF-α, capable of causing a 50% suppression of nodule number, cause a 50% suppression of nodules in the presence of IGF-I (not shown). Thus, IGF-I is not able to reverse the effect of TNF-α.

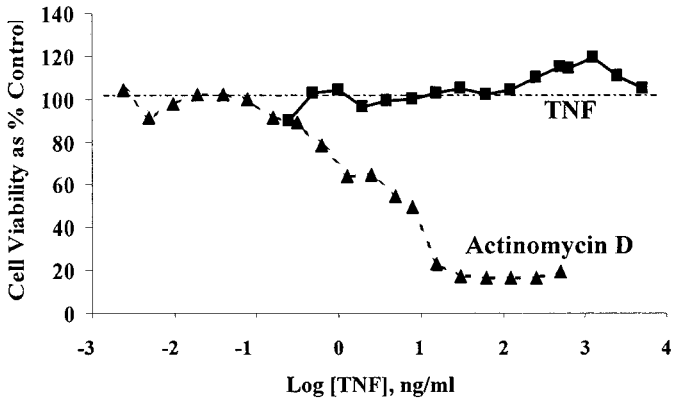

FIG. 6. TNF-α is not cytotoxic to MC3T3-E1–14 cells. TNF-α (0.244–5000 ng/ml) or the positive cytotoxic control, Actinomycin D (0.00244–500 ng/ml), were prepared in culture medium on 96-well plates. 10^4 cells/well were plated and grown for 4 days. Cell growth was assessed by measuring the reduction of Alamar Blue during an additional incubation of 6 h as described in *Materials and Methods*.

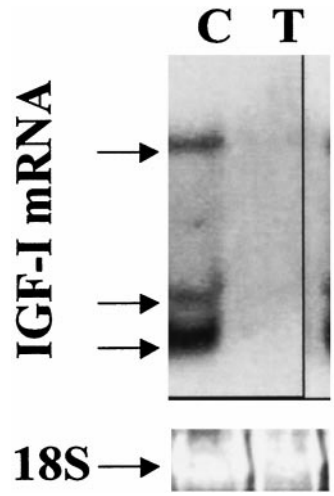

FIG. 7. TNF decreases steady-state IGF-I mRNA in fetal calvaria cultures. TNF-α (100 ng/ml) was added to fetal calvaria cultures on day 7. Cells were lysed with guanadinium isothiocyanate 16 h after TNF treatment and total RNA was purified. A full-length rat IGF-I cDNA probe was used to determine steady-state mRNA levels of IGF-I as described in *Materials and Methods*. Detectable IGF-I mRNA species are indicated by the *top 3 arrows*. 18S, 18S ribosomal RNA in the ethidium bromide-stained gel.

TABLE 2. Effect of TNF (10 ng/ml) on mRNA expression of genes known to promote osteoblastogenesis

Gene	% of Baseline mRNA level
IGF-I	<5
BMP-2	143
BMP-6	104
LMP-1	105

Results show mRNA as % baseline, 16 (IGF-I) or 24 (BMP-2, BMP-6, and LMP-1) hours after treatment with TNF-α. Values for mRNA levels are corrected for GAPDH mRNA on a Northern analysis of IGF-I. Values for BMP-2, BMP-6, or LMP-1 are corrected for 18S ribosomal RNA in the semiquantitative RT-PCR. The experiments for IGF-I were repeated three times, twice for BMPs, and once for LMP-1. In addition, mRNA levels were not consistently changed at later time points for BMPs or LMP-1.

Although mRNAs for BMPs are not reduced by TNF-α, we considered that TNF could cause resistance to the action of BMPs. To test this, BMP-6 was added to calvaria cultures from day 7 to day 14 with or without TNF-α. Figure 9 shows that BMP-6 treatment is osteogenic but is unable to reverse the inhibition by TNF-α (10 ng/ml). In addition, treatment of cultures with BMP-2 or -4 also results in a robust osteogenic response that is inhibited by TNF. In this experiment, TNF (10 ng/ml) was added simultaneously with BMP-2 or -4 (50 ng/ml) from days 7–14. Results were: Control 100 ± 2%, TNF 5.6 ± 1%; BMP-2 185 ± 3%, BMP-4 173 ± 2%, TNF+BMP-2 30 ± 3%, TNF+BMP-4 15 ± 2%) (n = 5–6/group, all groups differ from control, $P < 0.05$).

Discussion

Our data show that TNF-α dose-dependently inhibits differentiation of osteoblasts from their precursor cells in both

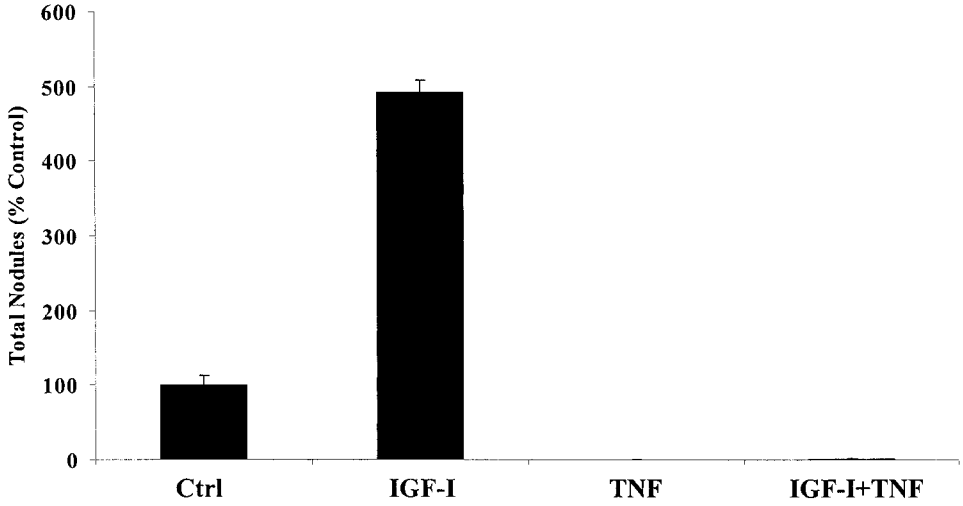

FIG. 8. IGF-I does not prevent TNF-α inhibition of differentiation in fetal calvaria cultures. TNF-α (100 ng/ml) and IGF-I (300 ng/ml) were added to cultures as indicated on days 7, 11, 14, and 18. Total nodules were determined on day 21. Data are mean ± SEM, n = 6. All groups differ from Control by ANOVA, $P < 0.05$. Control, No TNF added.

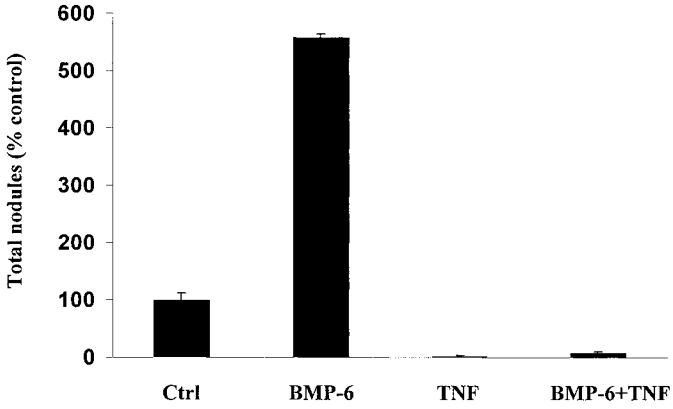

FIG. 9. BMP-6 does not prevent TNF-α inhibition of differentiation. TNF-α (10 ng/ml) and BMP-6 (42 ng/ml) were added to fetal calvaria cultures as indicated on days 7 and 11 and removed on day 14. Total nodules were counted on day 21. Data are mean ± SEM, n = 3–4. All groups differ from Control by ANOVA, $P < 0.05$. Control, No TNF added.

a fetal rat calvaria model and in clonal MC3T3-E1–14 cells. This effect of TNF-α is supported by the inhibition of mineralized nodule formation and inhibition of expression of the skeletal-specific gene, osteocalcin. Low doses of TNF-α are able to suppress differentiation, suggesting that osteoblast precursors are quite sensitive to the action of this cytokine. The rat calvaria model also allowed us to determine that a restricted time of TNF-α sensitivity occurs between days 7–10 of culture, when cells are postconfluent. This time period corresponds to a critical window of phenotype selection in these cultures of pluripotent precursor cells. In the postconfluent culture, osteoblast-specific factors allow the selection of the osteoblast over that of adipocytic, myocytic, or fibroblastic phenotypes. The inhibitory action of TNF-α is not associated with cytotoxicity of fetal calvaria cells or of MC3T3-E1–14 cells as determined by trypan blue exclusion and Alamar Blue reduction. In addition, careful counting of bone nodules in culture following TNF-α treatment did not reveal a loss of nodules once they are formed. Thus, TNF-α appears to block entrance into the osteoblast differentiation pathway rather than cause decreased survival of mature osteoblasts, once formed. We cannot completely exclude the possibility that TNF-α increases apoptosis of osteoblast progenitors as previously described (31). However, the lack of cell death in our cultures makes an apoptotic mechanism less likely.

We considered that TNF-α could inhibit differentiation by suppression of type I collagen synthesis, an important constituent of a competent skeletal matrix. The importance of type I collagen has been demonstrated with ascorbate depletion in this model (17, 40, 41, 44). Osteoblast differentiation, which is normally blocked by ascorbate depletion, will occur in the absence of ascorbate if cells are grown on plates precoated with type I collagen. In our hands, provision of a matrix replete with collagen (coated plates) does not prevent TNF-α inhibition of differentiation. Thus, TNF-α inhibition of collagen synthesis alone cannot explain inhibition of differentiation.

We determined whether factors known to augment differentiation were inhibited by TNF-α. In this report, we studied IGF-I expression, which is potently inhibited by TNF-α, and the osteogenic proteins BMP-2, BMP-6, and LMP-1. Addition of IGF-I to the medium in postconfluent cultures increases the formation of nodules, but TNF-α continues to inhibit this process. Similarly, response to osteogenic BMPs -2, -4, and -6 is inhibited by TNF-α. Thus, TNF-α blocks differentiation at a site distal to the action of IGF-I and BMP-6. We have previously shown that BMP-6 is one of the earliest BMPs to be expressed during differentiation and we cannot completely exclude an inhibitory effect of TNF on the response to BMP-2 or -4, which follow BMP-6 expression (27). In addition, TNF-α could inhibit the expression or response to osteogenic transcription factors induced by BMPs (SMADS) or to factors that select a skeletal specific path of differentiation (Cbfa-1, OSF-1 factor) (28, 29, 45, 46). Further work will be needed to address the possible actions of TNF-α at these levels.

It is possible that TNF-α could select precursor cells for differentiation along an adipocytic, fibroblastic, or skeletal muscle pathway, thus shunting cells away from an osteoblastic direction. However, the doses of TNF-α (1–10 ng/ml, days 7–14) that can completely suppress nodule formation in the fetal calvaria cell model are not associated with a change

in cell morphology. There are currently no reports of TNF-α induction of adipocyte, skeletal muscle, or fibroblastic differentiation; indeed, TNF-α has been shown to inhibit adipocyte differentiation (47–49). Thus, it is unlikely that TNF shunts precursor cells toward an alternate mature phenotype. We favor the hypothesis that TNF arrests differentiation by blocking transition of precursor cells into the differentiation pathway, perhaps by eliminating responsiveness to skeletal specific stimuli that are important at a stage later than BMP expression.

In summary, we have shown that TNF-α inhibits osteoblast differentiation. Suppression of osteoblast differentiation is likely to be an important mechanism of decreased bone formation in many circumstances where excess TNF-α is produced in the bone microenvironment. These include chronic inflammatory disease, estrogen deficiency, and some types of malignancy (50–53). Up-regulation of the TNF-stimulated transcription factor, NFκB, may also influence expression of additional regulators of both osteoblastogenesis and osteoclastogenesis (54, 55). The inhibitory action of TNF-α may occur at a point in the differentiation pathway distal to IGF-I, BMP-6, or LMP-1 expression. Further work will be needed to determine the specific mechanism of TNF-α action.

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