The p55 TNF receptor mediates TNF inhibition of osteoblast differentiation independently of apoptosis

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Submitted 2 November 2004; accepted in final form 23 December 2004

Gilbert, Linda C., Janet Rubin, and Mark S. Nanes. The p55 TNF receptor mediates TNF inhibition of osteoblast differentiation independently of apoptosis. Am J Physiol Endocrinol Metab 288: E1011–E1018, 2005. First published December 29, 2004; doi:10.1152/ajpendo.00534.2004.—After menopause, increased tumor necrosis factor-α (TNF-α) promotes bone resorption while inhibiting differentiation of new bone-forming osteoblasts (OB). TNF receptors, p55 and p75, signal similar intracellular pathways, but only p55 activates apoptosis. To evaluate the relationship between the TNF receptor mediating inhibition of OB differentiation and the role of apoptosis, narrow stromal cells (MSC) were cultured from mice deficient in either or both receptors. Cells grown in ascorbate and β-glycerophosphate produce alkaline phosphatase and osteocalcin and mineralize matrix. Treatment of wild-type or p55+/−/p75−/− MSC with murine TNF (binds p55 and p75) or human TNF (binds only p55) inhibited OB differentiation. TNF did not inhibit OB differentiation in p55−/− MSC. Expression of p75 modestly attenuated sensitivity to TNF. To determine the role of apoptosis, changes in total DNA, cell viability, caspase 3, and percentage of annexin V-positive cells were measured in MSC and preosteoblastic MC3T3 cells. TNF treatment that reduced differentiation by 50% did not decrease cell viability or increase apoptosis, as determined by alamar blue reduction, trypan blue exclusion, and percentage of annexin V-positive cells. TNF increased caspase 3 activity 1.5-fold in MC3T3 and significantly in MSC cells compared with 4-fold after 4 h actinomycin D. Treatment of MSC or MC3T3 cells with three caspase inhibitors failed to reverse the inhibitory effect of TNF on OB differentiation despite inhibition of caspase activity. These results suggest that the p55 receptor is essential, and p75 dispensable, for TNF inhibition of OB differentiation through a mechanism that does not require apoptosis.

osteoblast; p75; tumor necrosis factor receptor 1; tumor necrosis factor receptor 2; bone; osteoporosis; tumor necrosis factor; apoptosis

TUMOR NECROSIS FACTOR-α (TNF-α) is a major contributor to bone pathophysiology through stimulation of bone resorption and inhibition of bone formation (30). Elevated levels of TNF occur after menopause, a time associated with accelerated bone loss (18, 32). In mature osteoblasts (OB), TNF inhibits expression of genes by mature OB, including matrix proteins, IGF-I, and receptors for parathyroid hormone and vitamin D while simultaneously stimulating expression of OB genes that are osteoclastogenic signals, such as colony-stimulating factor-1, interleukin 6, receptor activator of NF-κB ligand, and its receptor (30, 41). In addition to compromising function of mature OB, TNF inhibits recruitment of new OB from their pluripotent precursors (1, 11, 12). Low-dose TNF treatment throughout a 14-day culture, or brief treatment during a critical period of phenotype selection, irreversibly prevents the appearance of OB in primary cultures of fetal rat calvaria or clonal murine MC3T3 preosteoblasts (11). This inhibitory effect is associated with suppression of transcription of the nuclear proteins, RUNX2 and osterix, which are required for OB differentiation and the formation of a mineralized skeleton (22, 25, 29).

TNF action begins with binding of the trimerized TNF protein to one of two cell surface receptors, p55 (TNFR1, TNFRSF1A) or p75 (TNFR2, TNFRSF1B), which are responsible for signal transduction (9). Both forms of the TNF receptor are widely expressed in tissues, including bone (23). Both p55 and p75 share signal pathways that activate nuclear entry of the transcription factor NF-κB and stimulation of mitogen-activated protein kinases (MAPks). An important difference between the p55 and p75 receptors is that the conserved TNF receptor family death domain is limited to p55. Association of the death domain with cytosolic adapter proteins, including FADD, activates the caspase cascade and causes mitochondrial membrane leakage, DNA cleavage, and cell death. Thus, depending on the receptor activated, apoptosis may be induced or excluded as a part of the downstream response. We considered that TNF signaling via p55 could stimulate apoptosis of preosteoblasts in addition to blocking their recruitment through suppression of RUNX2 and osterix.

Studies by Abbas et al. (1) showed that the p55 receptor was required for TNF inhibition of RUNX2 expression and OB differentiation. An important question is whether p55 signals the inhibition of differentiation by activation of apoptosis in precursor cells. Additionally, we considered that p75, although dispensable for inhibition of differentiation, might modify the TNF/p55 response. To answer these questions, we used narrow stromal cells (MSC) derived from mice deficient in one or both receptors and clonal preosteoblast MC3T3 cells. In addition, we determined the contribution of p55-mediated apoptosis in TNF action by blocking the apoptotic response in TNF-treated cells. Here we present data supporting the hypothesis that inhibition of OB by TNF requires only the p55 receptor; however, apoptosis is not required.

MATERIALS AND METHODS

Materials. Minimal essential medium (MEM)-α was from GIBCO/Invitrogen (catalog no. 12571-063; Grand Island, NY). Fetal bovine serum was from HyClone Laboratories (Logan, UT), and D-ascorbate and β-glycerophosphate were purchased from Sigma Chemical (St. Louis, MO). Human (h) and murine (m)TNF were from PeproTech (Rocky Mountain Center, Denver, CO). L-Ascorbate was from Calbiochem (La Jolla, CA), and osteocalcin was from R&D Systems (Minneapolis, MN).

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Hill, NJ). Annexin V (FITC and propidium iodide kit) was obtained from BD Biosciences (Palo Alto, CA). The cell-permeable caspase inhibitors DQMD (Z-DQMD-FMK, caspase 3 inhibitor V), DEVD (Z-DEVD-FMK, caspase 3 inhibitor), and VAD (Z-VAD-FMK, caspase inhibitor I) were obtained from Calbiochem (La Jolla, CA).

Cell culture. The Emory University and Veterans Affairs Medical Center animal use committee approved all procedures. Mouse strains carrying homozygous “knockout” mutations in the TNF receptors and the corresponding control strains were purchased from Jackson Laboratories (Bar Harbor, ME). The background strains for the p55 and p75 knockouts, although both C57BL/6J, were studied as separate control groups to rule out minor background differences, since they had been inbred over time. The backgrounds associated with each knockout were as follows: p55 p75: C57BL/6J, stock no. 000664; p55 p75: C57BL/6J, stock no. 000664; p75 p75: B6; 129S2F2/J, stock no. 101045. The knockout stocks used in the study included the following: p55 p75: C57BL/6-TNFrsf1btm1imx, stock no. 003242; p75 p75: B6; 129S-TNFrsf11imx, stock no. 003246; B6; 129S2-TNFRsft11imx, stock no. 002620; and p55 p75 p75: B6; 129S-TNFRsft11imx, TNFRsf11imx, stock no. 003243.

Bone MSC cultures from mouse strains homozygous for knockout mutations in the TNFR1 gene (p55 p55), the TNFR2 gene (p75 p75), or both genes (p55 p75 p75) and their wild-type (WT) controls were established using typical methods. Briefly, femurs and tibias from individual mice were excised, and marrows were flushed with MEM-β-glycerophosphate (complete medium), diluted with an additional 10% FBS, 2.5 ml MEM-H9251/H11001 10% FBS and switched to complete medium the next day. Marrow cells were centrifuged at 1,000 g for 10 min, resuspended in 10 ml MEM-α, and recentrifuged. Cell pellets from individual mice were resuspended in 2.5 ml MEM-α + 10% FBS + 50 μg/ml l-ascorbate + 5 mM β-glycerophosphate (complete medium), diluted with an additional 10 ml of complete medium, and plated at 1 ml/well in 12-well plates.

Measurement of total DNA in the attached cells showed no difference between knockout and WT cells (within a strain) 5 days after plating (data not shown). In addition, preliminary data using more purified MSC, which had been replated as a secondary culture to assure the absence of residual T cells or macrophages, gave the same response to TNF as the above method. For the TNF dose-response experiments, cells were plated at 0.5 ml/well in 24-well plates. The plates were TNF as the above method. For the TNF dose-response experiments, absence of residual T cells or macrophages, gave the same response to (data not shown). In addition, preliminary data using more purified

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MC3T3-E1 (clone 14), a murine preosteoblast clonal cell line, was kindly provided by Dr. R. Franceschi (University of Michigan, Ann Arbor, MI). Stock cultures were grown in MEM + 10% FBS without ascorbate. For experiments, cells were plated in MEM + 10% FBS and switched to complete medium the next day.

Alkaline phosphatase assays. Alkaline phosphatase was determined by measuring the conversion of p-nitrophenyl phosphate to p-nitrophenol and measured by spectrophotometry at 405 nm after 15 min of incubation according to the manufacturer’s instructions (Sigma).

Osteocalcin assays. Osteocalcin levels in culture medium from individual wells were measured using a mouse osteocalcin immuno-radiometric assay kit from American Laboratory Products (Windham, NH). The assay service was provided by the Yerkes Endocrine Core Laboratory at Emory University.

Mineralization assays. Cultures were fixed with 70% ethanol and stained with the von Kossa silver stain for mineral (6). Quantitative assessment of mineralization was done by staining duplicate cultures with alizarin red on day 14. Mineral-bound alizarin red was eluted with 10% cetylpyridinium chloride (Sigma) and quantitated for triple cultures by spectrophotometry at 570 nm (7).

DNA assay. Cell lysates for DNA assay were obtained with M-PER (Pierce Biotechnology, Rockford, IL), and the double-stranded DNA was measured using the FluoroReporter Blue Fluorometric dsDNA Quantitation kit from Molecular Probes (Eugene, OR) according to the manufacturer’s instructions.

Cell viability assay. Alamar blue staining was done as previously described (11).

Annexin V assay. Annexin V-FITC and propidium iodide labeling of MC3T3 cells was analyzed using a fluorescence-activated cell sorter (FACS) Calibur according to the protocol provided with the ApoAlert Annexin V Apoptosis kit (BD Biosciences). Annexin V binding to translocated membrane phosphatidylserine was used as an indicator of apoptosis (27).

Caspase 3 assay. Caspase 3 activity in MSC or MC3T3 was measured in cell lysates by use of a colorimetric assay kit (Sigma). RT-PCR. PCR to detect expression of murine p55 (TNFR1, accession no. X57796) and p75 (TNFR2, accession no. M60649) receptors in MC3T3 cells were done using the following primers: p55 sense 5'-CCGGGCCACCTGGTCCG-3', antisense 5'-AAATGAATGTTC-CTTTGTG-3', p75 sense 5'-GTCGCCGCTGTCTTCAACTG-3', antisense 5'-GGTATACTAGCTTGCCTCAGTGC-3' (36).

Statistics. Results were analyzed using ANOVA. Differences between multiple groups were determined by the method of Tukey. Differences between a control WT culture and any one group were determined using the method of Dunnett. Statistics were done using Prism 3.03 software (GraphPad, San Diego, CA).

RESULTS

TNF inhibition of OB differentiation is mediated by p55. To determine which TNF receptors mediate inhibition of OB differentiation, we cultured MSC from mice deficient in p55, p75, or both receptors. The knockouts were derived from different background strains that revealed some difference in the mineralization of the differentiated cultures at the end of the 14-day culture period. Therefore, experiments on cells from any knockout were always done using receptor replete controls of the identical background. Cells were cultured for 14 days with l-ascorbate and β-glycerophosphate to induce differentiation. During the initial 3–4 days in culture, cells proliferate to confluence, after which they acquire the capacity to secrete a matrix rich in type I collagen, express alkaline phosphatase, form a patchy network of mineralized nodules, and secrete the skeletal specific matrix protein osteocalcin (26). Figure 1A, a and b, shows that addition of hTNF or mTNF (10 ng/ml, days 2–14) to WT marrow stromal cultures completely inhibited differentiation and formation of the mineral layer, as shown by von Kossa staining on day 14. This dose of TNF is well above that needed for a maximum inhibitory effect, previously shown to have an IC50 of 0.6 ng/ml and maximum action at 2 ng/ml in fetal calvaria cells and MC3T3 preosteoblasts (11). As expected, TNF had no effect on differentiation in cultures derived from p55 p75 p75 mice (Fig. 1A, a and b).

The response to TNF in cultures from p55 or p75 single knockouts is shown in Fig. 1B. MSC from p55 p75 homozygotes were completely unresponsive to TNF with regard to mineralization, as shown by both von Kossa and alizarin red staining and by assay for alkaline phosphatase and osteocalcin secretion (Fig. 1B, a, c, and e), similar to the p55 p75 p75 cultures. In contrast, TNF inhibited these markers of the OB phenotype in cultures from p75 p75 mice (Fig. 1B, d, and f).
Fig. 1. A: murine (m) and human (h) tumor necrosis factor (TNF)-α inhibit differentiation of murine MSC toward the osteoblast phenotype. MSC from TNF-α receptor double knockout mice (p55⁻/⁻ p75⁻/⁻) or wild-type (WT) mice of identical background were grown for 14 days as described in MATERIALS AND METHODS. mTNF-α (a) or hTNF (b), 10 ng/ml, was added on days 2, 5, 7, 9, and 12 in fresh medium. Cultures were fixed with 70% ethanol on day 14 and stained for mineral by the von Kossa method. C, controls. B: TNF inhibition of osteoblast differentiation requires the p55 receptor. Marrow stromal cell (MSC) cultures were established as in A. Cells were plated day 0, and TNF (10 ng/ml) was added beginning on day 2. Medium samples were collected from each well on day 14 and stored at −70°C for osteocalcin assays. Cultures were fixed with 70% ethanol on day 14 and stained for mineral by the methods of von Kossa or alizarin red staining. Cultures from p55⁻/⁻ mice are resistant to the effect of TNF, as seen by mineralization on day 14 (a), alkaline phosphatase activity (c), and secretion of osteocalcin (e) in the presence of TNF. MSC from p75⁻/⁻ mice remain susceptible to TNF inhibition, as seen by lack of mineralization (b) and absence of alkaline phosphatase (d) and osteocalcin (f). C, control; WT, wild-type MSC from matched background of the knockout strain. *P < 0.05 by ANOVA. The results shown were obtained using hTNF. mTNF treatment gave identical results (data not shown). C: p75 reduces sensitivity to mTNF. mTNF or hTNF was added at concentrations from 5 to 10 ng/ml continuously from day 2. a: Complete suppression of differentiation with 5 or 10 ng/ml mTNF in p75⁻/⁻ MSC. Residual differentiation is still seen in the WT MSC (p75⁻/⁻) at the same dose. b: Identical dose-response effect with WT and p75⁻/⁻ MSC with hTNF, which binds only the p55 receptor.
The latter results were not a strain effect, since p75−/− mice from an additional background yielded identical results (data not shown).

To distinguish the role of receptor types, we also took advantage of the selective receptor binding properties of mTNF (binds p55 and p75) and hTNF (binds p55 exclusively). Cultures from p75−/− mice and WT of identical background were treated with m- or hTNF at doses from 0.1 to 10 ng/ml. Figure 1C, a and b, shows that p75−/− cells were more sensitive to mTNF than p75+/− cells, as seen by the residual mineralization at doses of 5 and 10 ng/ml mTNF in the p75−/− cells (Fig. 1C, a and b). The protective effect of p75 was not observed with hTNF treatment, since hTNF signals exclusively through the p55 receptor in murine MSC. p55−/−/p75+/− cells do not respond to either hTNF or mTNF (data not shown). Some between-background and between-experiment differences in mineralization were noted using von Kossa; however, within any one experiment, control and TNF-treated cells were derived from the same background.

Apoptosis is not required for TNF inhibition of OB differentiation. To determine the role of p55-mediated apoptosis, we measured effects of TNF on total DNA, cell viability, and caspase 3 activity in WT MSC and in preosteoblastic MC3T3 cells. In addition, annexin V binding was measured by FACS in MC3T3 cells. For WT MSC derived from the inbred p55 background, TNF treatment caused a slight but significant increase in total DNA (Fig. 2A, C-wt55 vs. T-wt55). This increase was abolished by p55 knockout (C-ko55 vs. T-ko55). TNF did not cause an increase in total DNA in WT MSC derived from the p75 control background. A decrease in total DNA was seen in response to TNF treatment of p75−/− MSC (C-ko75 vs. T-ko75).

Cell viability was determined using alamar blue. In apoptotic cells, oxidized blue alamar is retained rather than reduced to a pink color by viable functioning mitochondria. Cell viability was maintained in WT MSC treated with TNF (10 ng/ml) from days 2 to 7 or from days 5 to 7, a dose that inhibits OB differentiation (Fig. 2B).

We previously showed that differentiation of the clonal cell line, MC3T3, is inhibited by TNF treatment. TNF treatment, up to 1,000 ng/ml for 48 h, does not reduce viability in these cells, as measured by alamar blue assay (11). Because this cell line expresses p55 in addition to p75 receptors (Fig. 2D), we determined whether continuous exposure to TNF caused apoptotic cell death. Figure 2C shows that trypan blue exclusion, as measured on days 3 and 7 of culture, is maintained in cells treated continuously with 10 ng/ml TNF. A decrease in trypan blue exclusion was observed only on day 14, after differentiation was complete (Fig. 2C).

Figure 3 shows the results for assays of caspase 3, the terminal caspase activated in the apoptotic cascade. Figure 3A shows that treatment of MC3T3 cells with TNF increased caspase 3 activity only slightly. This increase was blocked by cotreatment with the different caspase inhibitors DQMD, VAD, and DEVD. Actinomycin D stimulated caspase 3 more robustly, an effect also blocked by the caspase inhibitors (Fig. 3A). In MSC, TNF (10 ng/ml, days 2–7 or 5–7) did not significantly increase caspase activity (Fig. 3B). Actinomycin D, used here as a positive control, caused a large increase in caspase 3 activity, as expected. Assays of caspase 8 activity were also done in the same experiments and did not show a significant increase after TNF stimulation for 48 h (from days 5 to 7) in MSC (control 5.49 ± 1.35, TNF 6.00 ± 1.01, actinomycin D 9.56 ± 1.22 μmol p-nitroaniline·min−1·μg−1·109, data not shown).

FACS was used to determine the percentage of MC3T3 cells undergoing apoptotic cell death after treatment with TNF. Table 1 shows the percentage of cells bound by annexin V, after different treatments with TNF or TNF plus actinomycin D. The protective effect of p75 was not observed with hTNF treatment, since hTNF signals exclusively through the p55 receptor in murine MSC. p55−/−/p75+/− cells do not respond to either hTNF or mTNF (data not shown). Some between-background and between-experiment differences in mineralization were noted using von Kossa; however, within any one experiment, control and TNF-treated cells were derived from the same background.

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Actinomycin D, 4 h 8.08 19.96

TNF, 48 h

0.05 by ANOVA.

A. alizarin red stain; Fig. 4 (100 ng/ml, 4 h) was used as a positive control. pNA, p-nitroaniline. B: caspase 3 activity in MSC cells after hTNF treatment (10 ng/ml) on days 5–7. *P < 0.05 by ANOVA.

(Propidium iodide) in the TNF group at 48 h was not significant (TNF 6.94% vs. control 5.80%).

Cell permeable caspase inhibitors were used to determine if the inhibition of OB differentiation in MC3T3 cells was caused by caspase-induced apoptosis (Fig. 4). Cells were treated with TNF in the presence of the caspase inhibitors DQMD or DEVD, and mineralization of matrix was measured on day 16 (Fig. 4A, alizarin red stain; Fig. 4B, alizarin quantitation after extraction). TNF (10 ng/ml) treatment was limited to days 3–6 in this experiment to increase the sensitivity of detecting a reversal of inhibition by the caspase inhibitors. Alizarin red staining for mineral showed the expected differentiation in the control groups (C) and the inhibitory effect of TNF treatment from days 1 to 16 (94.3 ± 0.1% inhibition) or days 3–6 (59.2 ± 1% inhibition). Neither the DMSO vehicle alone nor the caspase inhibitors at 1 or 10 μM (DQMD, DEVD) affected basal differentiation of the cells. It was shown that TNF (days 3–6) continued to inhibit differentiation of the MC3T3 cells by up to 38–52% in the presence of the caspase inhibitors at doses previously shown to be effective in preventing actinomycin D-induced cell death (Table 1). A similar experiment using TNF on days 1–16 gave identical results (data not shown).

Table 1. Fluorescence-activated cell sorter analysis of annexin V binding in MC3T3 cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Annexin V, %</th>
<th>Propidium Iodide, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.02</td>
<td>5.80</td>
</tr>
<tr>
<td>TNF, 48 h</td>
<td>1.27</td>
<td>6.94</td>
</tr>
<tr>
<td>TNF, 48 h + DQMD</td>
<td>3.40</td>
<td>9.43</td>
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<tr>
<td>Actinomycin D, 4 h</td>
<td>8.08</td>
<td>19.96</td>
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<tr>
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DISCUSSION

Our results indicate that the p55 TNF receptor is required and sufficient for the inhibitory effect of TNF on OB differentiation. These conclusions were based on data showing that both mTNF and hTNF inhibit differentiation in MSC cells. This effect of TNF was abolished in p55−/− mice. Our results confirm those first reported by Abbas et al. (1). These investigators showed that primary stromal cells derived from TNFR1 knockout mice (p55−/−) failed to respond to TNF with inhibition of β-glycerophosphate-induced expression of markers of the OB phenotype, including nuclear RUNX2 DNA binding and alkaline phosphatase activity. Here, we confirm that p75 knockout is not required for TNF action, although this receptor does influence cell sensitivity to TNF, as shown by a shift in the TNF dose–response curve. These data suggest that p75 modulates the response to TNF in OB, as suggested for other cell types, although the effect is small (43, 47).

P55 and p75 are widely expressed receptors that signal redundant and unique pathways (35, 45). Both receptors stimulate gene transcription by activating NF-κB and MAPK, signals that activate transcriptional products regulating bone resorption and bone formation (30). We considered that TNF might inhibit OB differentiation by causing a p55-specific...
apoptosis of preosteoblasts or possibly a shortened life span of the newly formed mature OB (17). Either effect would eliminate the steady-state pool of functioning OB. TNF has been shown to cause apoptosis of mature OB in culture; however, this effect of TNF is dependent on cell type and culture conditions (5, 16, 19, 21, 34). C3C12 cells, which can be induced to differentiate along the OB pathway, are highly sensitive to the apoptotic effect of TNF (10). TNF-induced apoptosis is revealed by cotreatment of OB with cycloheximide or by reduction of medium serum concentration. In our hands, the IC50 for TNF inhibition of OB differentiation was 1–2 ng/ml for MSC and 0.5 ng/ml for MC3T3 or fetal rat calvaria cells, doses below that used to demonstrate an apoptotic response in most studies. In addition, we cultured cells at increased density in the presence of ascorbate and β-glycerophosphate, factors that may have protected cells from apoptosis secondary to autocrine production of IGF-I (46). Bone morphogenetic proteins (BMP), which induce OB differentiation, stimulate apoptosis; however, BMP-stimulated differentiation can occur independently of apoptosis in Saos-2 and OHS4 cells treated with caspase inhibitors (15). In contrast, Mogi and Togari (28) recently showed that caspase inhibition blocked BMP-4-stimulated differentiation in MC3T3 cells, suggesting that apoptosis was required for the differentiation program to proceed normally.

Our results showed that apoptosis is not part of the mechanism through which TNF inhibits differentiation. Cell viability was maintained in TNF-treated cultures as measured by alamar blue reduction or trypan blue exclusion. In addition, TNF did not decrease cell number in WT or p55−/− stromal cells, as measured by total DNA per well. In fact, a small increase in cell number occurred after TNF treatment in WT stromal cultures from the p55 background, an effect previously observed by us in ROS 17/2.8 osteoblastic cells (data not shown). Interestingly, p75−/− stromal cells showed a small decrease in cell number after TNF treatment. Nevertheless, there was no concordance between changes in cell number and p55 mediation of TNF action.

We used flow cytometry to determine the percentage of phosphatidylserine-positive cells as determined by annexin V binding. Our data showed that, under our conditions of culture, only 3% of MC3T3 cells bind annexin V and that TNF treatment did not increase this population after 48 h. We also measured caspase 3, the terminal member of the caspase cascade. TNF treatment increased caspase 3 modestly in MSC and insignificantly in MC3T3 cells, consistent with the annexin V results. For all of these experiments, brief actinomycin D treatment, used as a positive control, decreased cell viability and increased caspase 3 and annexin V, as expected. Finally, we showed that the minimal level of apoptosis occurring in our cultures was unlikely to be affecting the rate of OB differentiation. Treatment with caspase inhibitors, which reversed part or all of the actinomycin D increase in caspase 3 and annexin V binding, did not block the inhibition of OB differentiation. We did not observe an effect of caspase inhibitors alone on differentiation of MC3T3 cells, in contrast to the study of Mogi and Togari (28). As noted above, culture conditions were not identical to those used in other studies.

Each TNF receptor type imparts specific biological responses. Unique responses attributed to p55 include cytotoxicity, prevention of early arteriosclerosis, stimulation of liver regeneration, B cell follicle development, autoimmune β-cell toxicity, and the mediation of bacterial lipo polysaccharide-induced sepsis (14, 20, 33, 38, 40, 44, 48). In contrast, thymocyte development and dermal hypersensitivity after ultraviolet B or TNF exposure have been attributed to signaling via p75 (39, 42, 50). Several studies have shown that p55 signals osteoclast recruitment and bone loss in a variety of conditions, in addition to the suppression of RUNX2 activation in preosteoblasts, a prerequisite for their differentiation (1–4, 8, 37, 49). Thus our data on OB differentiation are consistent with prior reports related to osteoclast differentiation.

Our results suggest that the major effect of TNF is to prevent recruitment of OB in the steady-state pool of functioning bone-forming cells. Activation of NF-κB by TNF suppresses expression of the RUNX2 and osterix transcription factors, without which precursor cells do not differentiate to the OB phenotype (12, 13, 22, 25, 29). p75 is known to signal the same nonapoptotic pathways as p55, including NF-κB and MAPK. Since p75 alone was unable to support the inhibitory effect of TNF, then p55 may activate an additional pathway regulating the differentiating transcription factors. Alternatively, the p75 stimulation of NF-κB or MAPK in marrow cells may be weaker than that from p55, as previously suggested (2). Thus TNF may have been unable to inhibit OB differentiation in the p55−/−p75−/+ MSC because the NF-κB activation was inadequate or a separate p55-mediated pathway was required. Further work will be needed to determine the p55 signal pathway important for regulation of differentiation.

TNF inhibition of OB differentiation may contribute to the blunted bone formation response that occurs at the time of estrogen withdrawal at menopause. The elevated TNF production at that time stimulates osteoclastogenesis and increased bone resorption. Although some increase in bone formation has been described with estrogen withdrawal, it is inadequate to counter the increased resorption, leading to a net bone loss over time. Our results suggest that TNF could contribute to the inadequate coupling of formation and resorption during menopause by blocking recruitment of new OB in the pool of bone-forming cells. In addition, TNF is known to impair matrix protein production and cause vitamin D resistance in mature OB (30).

Our data did not address the possibility that a ligand other than TNF-α could signal via the p75 receptor, leading to a more robust response and potential regulation of OB differentiation through this receptor. In the mouse, the p75 receptor is bound by lymphotoxin-α (LT-α, TNF-β) in addition to TNF-α, whereas p55 is bound only by TNF-α. In bone, paracrine LT-α can further stimulate TNF-α production (31). Thus our data exclude a role for p75 with regard to TNF stimulation or in basal conditions, but does not address a potential role of LT-α or an unknown p75 ligand.

In summary, TNF-α inhibition of OB differentiation is mediated by the p55 TNF receptor, which is required and sufficient for this biological effect. This action of p55 does not involve apoptosis.

ACKNOWLEDGMENTS

We thank Xiaofei He for technical assistance.
GRANTS
This work was supported by National Institute of Arthritis and Musculo-
skeletal and Skin Diseases Grant RO1 AR-46452 and by funds from the
Department of Veterans Affairs (Merit Review) to M. S. Nanes.

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