Tumor Necrosis Factor-α Promotes In Vitro Calcification of Vascular Cells via the cAMP Pathway

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**Background**—Vascular calcification is an ectopic calcification that commonly occurs in atherosclerosis. Because tumor necrosis factor-α (TNF-α), a pleiotropic cytokine found in atherosclerotic lesions, is also a regulator of bone formation, we investigated the role of TNF-α in in vitro vascular calcification.

**Methods and Results**—A cloned subpopulation of bovine aortic smooth muscle cells previously shown capable of osteoblastic differentiation was treated with TNF-α, and osteoblastic differentiation and mineralization were assessed. Treatment of vascular cells with TNF-α for 3 days induced an osteoblast-like morphology. It also enhanced both activity and mRNA expression of alkaline phosphatase, an early marker of osteoblastic differentiation. Continuous treatment with TNF-α for 10 days enhanced matrix mineralization as measured by radiolabeled calcium incorporation in the matrix. Pretreatment of cells with a protein kinase A–specific inhibitor, KT5720, attenuated cell morphology, the alkaline phosphatase activity, and mineralization induced by TNF-α. Consistent with this, the intracellular cAMP level was elevated after TNF-α treatment. Electrophoretic mobility shift assay demonstrated that TNF-α enhanced DNA binding of osteoblast specific factor (Osf2), AP1, and CREB, transcription factors that are important for osteoblastic differentiation.

**Conclusions**—These results suggest that TNF-α enhances in vitro vascular calcification by promoting osteoblastic differentiation of vascular cells through the cAMP pathway. (*Circulation. 2000;102:2636-2642.*)

**Key Words:** muscle, smooth ■ signal transduction ■ atherosclerosis

Tumor necrosis factor-α (TNF-α), a pleiotropic cytokine, has been shown to play a role in both vascular and bone pathophysiology. TNF-α is mainly secreted by macrophages in response to factors such as oxidized LDL,1 acetylated LDL,2 physically damaged extracellular matrix,3 or bacterial infection.4 TNF-α influences many aspects of atherogenesis,5 including increasing permeability of endothelial cells,6 promoting monocyte adhesion,7 inducing macrophage differentiation,8 and promoting foam cell formation.9 In vivo, TNF-α induces arteriosclerosis-like lesions in coronary arteries.10 TNF-α also regulates bone turnover, inhibiting osteoblastic function11–13 and stimulating bone resorption.14

Vascular calcification is a pathological condition that occurs in many diseases, including atherosclerosis, diabetes, and uremia.15 We and others have provided evidence that atherosclerotic calcification resembles osteogenesis,16–18 and factors regulating bone mineralization have been demonstrated in calcified atherosclerotic plaques.16,17,19–22 In previous studies of vascular calcification, we cloned a subpopulation of vascular cells from the bovine aortic media.16,17 In long-term culture, these calcifying vascular cells (CVCs) express osteoblastic differentiation genes in the sequence reported for bone cells and form a mineralized matrix in vitro.23 Similar in vitro models have been developed by other investigators.18,24–26 Agents that are present in atherosclerotic arteries have been shown to promote the differentiation and mineralization of these vascular cells.17,27

Although TNF-α has been detected in both human and mouse atherosclerotic lesions,28,29 its contribution to vascular calcification has not been assessed. We and others found TNF-α immunoreactivity in arteries with calcification in C57BL/6J mice fed an atherogenic diet.29 In contrast, TNF-α is not found in normal vascular intima or fatty streaks.29,30 Additional evidence that TNF-α may regulate vascular calcification arises from recent in vivo studies showing that mice lacking the osteoprotegerin gene, a novel soluble member of the TNF-α receptor superfamily, develop vascular calcification in addition to premature osteoporosis.31 Therefore, we hypothesized that TNF-α regulates calcification in atherosclerotic lesions, and we examined the direct role of TNF-α in in vitro vascular calcification.

In the present report, CVCs were treated with TNF-α and the effects of TNF-α on differentiation and mineralization of CVCs were determined. The results indicate that TNF-α promotes CVC mineralization by modulating the expression of genes important for extracellular matrix formation and mineralization. We also showed that the enhanced mineralization of CVCs by TNF-α was mediated in part by activation...
of the cAMP pathway and enhancement of the activity of transcription factors that are important for osteoblastic differentiation.

**Methods**

**Materials**

Human recombinant TNF-α was purchased from Sigma Chemical Co. [45Ca]Cl2, [α-32P]dCTP, [γ-32P]ATP, and [3H]thymidine were from Amersham. Oligonucleotide probes containing AP1 and cAMP-responsive element-binding protein (CREB) consensus sequences were purchased from Santa Cruz Biotechnology, Inc. Oligonucleotide probe containing Cbfa1 binding site (OSE2) from osteocalcin promoter was synthesized by Gibco BRL. Pertussis toxin, chelerythrine chloride, phorbol 12-myristate 13-acetate (PMA), AACOCF3, indomethacin, and KT5720 were purchased from Calbiochem. Anti-TNF-α antibody was obtained from BioSource International. Anti-Fos and anti-Jun antibodies were purchased from Santa Cruz Biotechnology, Inc. Antibody specific to Cbfa1/Osf2 was a generous gift from Dr. Gerald Karsenty. Quantification of bacterial endotoxin in human recombinant TNF-α was tested with quantitative chromogenic limulus amebocyte lysate (LAL; BioWhittaker, Inc).

**Cell Culture**

CVCs were isolated from bovine aortic media layer and were identified as described previously.16,17 CVCs were grown in Dulbecco’s modified Eagle’s medium (DMEM; Irvine Scientific) containing 15% heat-inactivated FBS (HyClone Labs) and supplemented with sodium pyruvate (1 mmol/L), penicillin (100 U/mL), and streptomycin (100 U/mL), all from Irvine Scientific. Cells (passages 12 to 17) were treated at 80% confluence with appropriate agents in DMEM containing 5% FBS.
Alkaline Phosphatase Activity Assay
Cells were cultured in 24-well plates and treated with or without test agents at 80% confluence for 2 to 3 days. Cells were lysed and alkaline phosphatase (ALP) activity was measured as previously described.22 ALP activity was normalized to total protein determined with the Bio-Rad protein assay solution (Bio-Rad Laboratories).

Reverse Transcription–Polymerase Chain Reaction
Total RNA (3 μg) extracted as mentioned above was reverse-transcribed, and polymerase chain reaction (PCR) with ALP and GAPDH-specific primers was performed as described previously.23

45Ca Incorporation Assay
Cells were cultured in 24-well plates and treated at 80% confluence with or without 10 ng/mL TNF-α. In the experiments in which there were pretreatments, cells were treated with agents 2 hours before addition of TNF-α to the media. After 3 to 4 days of incubation, media were replaced with fresh media containing TNF-α (pretreated agents and TNF-α), 4 mmol/L CaCl2, and 5 mmol/L β-glycerophosphate (βGP) and incubated for an additional 3 to 4 days. Cells were washed 3 times and incubated in fresh media containing TNF-α, βGP, and [45Ca]CaCl2 (1.0 μCi/mL) for an additional 48 hours. The matrix-bound radiolabeled calcium incorporation was performed as described previously.23

cAMP Assay
Cells were plated in 6-well plates and treated at 80% confluence with serum-free medium for 30 minutes before being treated with 30 ng/mL TNF-α in serum-free medium supplemented with 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX; Calbiochem) for an additional 30 minutes. After incubation, cells were washed and scraped in 20 mmol/L phosphate buffer (pH 7.0) containing 20 mmol/L EDTA and 1 mmol/L IBMX. The extracts were sonicated briefly and boiled for 7 minutes to precipitate the cellular proteins. The extract was clarified by centrifugation, and supernatant was assayed for cAMP with the cAMP EIA kit (Stratagene).

Gel Mobility Shift Assay
Nuclear extracts were prepared as described previously.24 Three to 5 μg of nuclear extracts was incubated with binding buffer containing 20 mmol/L Tris-HCl (pH 8), 10 mmol/L NaCl, 3 mmol/L EDTA, 0.05% Nonidet P-40, 5 mmol/L dithiothreitol, 5% glycerol, and 20 mmol/L Tris-HCl (pH 8), 10 mmol/L NaCl, 3 mmol/L EDTA, and 1 mmol/L IBMX. The extracts were subjected to electrophoresis on a chilled 5% polyacrylamide gel at 400 V for 14 minutes. In the supershift experiments, respective antibodies were preincubated with nuclear extracts for 30 minutes before addition of the labeled probe. In competition experiments, 100-fold excess of the respective cold oligonucleotides was preincubated with nuclear extracts for 15 minutes before addition of the labeled probe.

Statistical Analysis
Data are expressed as mean±SD, and means were compared by 1-way ANOVA. The Tukey-Fisher least significant difference (LSD) criterion was used to judge statistical significance.

Results
Effects of TNF-α on Markers of Osteoblastic Differentiation and Mineralization
To determine the effects of TNF-α on onset of osteoblastic differentiation, CVCs were treated for 3 days, and 2 established markers of early osteoblastic differentiation, morphological change25 and ALP activity,26 were assessed. TNF-α induced a morphological change from an elongated to a cuboidal shape (Figure 1A). Treatment of CVCs with increasing concentrations of TNF-α showed a dose-dependent increase in ALP activity (Figure 1B). We compared the mean values with 1-way ANOVA. A significant increasing trend by dose was observed (F=2117; P<0.00005). The effect of TNF-α on ALP activity was not due to an artifact of normalization, because even without normalization for total protein, the ALP activity was increased 15-fold by TNF-α (10 ng/mL) (data not shown). Further tests were performed to determine that the effects were specific to TNF-α. Results from a quantitative chromogenic LAL assay showed an endotoxin (lipopolysaccharide) level of 4×10⁻³ pg/μL, which is 5 orders of magnitude below the level required for activity in this system. In addition, treatment with TNF-α antibody completely blocked the induction of ALP activity (data not shown), indicating that TNF-α effect on ALP activity is not attributable to contamination with bacterial endotoxin.

To determine whether the induction of ALP by TNF-α was at the level of mRNA expression, total RNA was isolated from samples treated with TNF-α for 3 days, and semiquan-
titative reverse transcription-PCR (RT-PCR) was performed. Results showed that TNF-α also enhanced mRNA expression of the ALP gene (Figure 1C). TNF-α did not enhance expression of the internal control gene GAPDH. Treatment of CVCs with TNF-α for 3 days did not appreciably affect expression of other osteoblastic genes, type I procollagen, and osteocalcin (data not shown).

To determine whether enhanced ALP activity ultimately results in increased mineralization, CVCs were treated with increasing concentrations of KT5720 before addition of TNF-α (10ng/mL) to media. A. Phase contrast (magnification ×40) of control, TNF-α alone, TNF-α + 5.0 μmol/L KT5720, or TNF-α + 10.0 μmol/L KT5720 (n=2). B, ALP activity measured 2 days after treatments. All samples contain 10 ng/mL TNF-α. Each bar represents mean of 4 wells (n=2). **P<0.001 vs TNF-α alone. C. 45Ca incorporation in matrix measured 9 days after treatment. All samples contain TNF-α. Each bar represents mean of 4 wells (n=2). **P<0.05 vs TNF-α alone. D. Growth-arrested CVCs were treated with TNF-α (50 ng/mL) in serum-free medium supplemented with IBMX for additional 30 minutes, and intracellular cAMP levels were analyzed. Average cAMP levels from duplicate wells are shown (n=2). **P<0.05 vs control.

Second Messenger of TNF-α in CVC Differentiation and Mineralization

In various cell types, the pleiotropic effects of TNF-α are mediated by different intracellular signaling pathways. Therefore, we examined the signaling pathway used by TNF-α in induction of CVC differentiation and mineralization. First, we assessed the role of pertussis toxin–sensitive G protein, which mediates TNF-α signaling in other systems.5,37 CVCs were pretreated with pertussis toxin for 2 hours before TNF-α treatment. Results showed that pertussis toxin did not inhibit TNF-α–induced ALP activity (measured 2 days after TNF-α treatment), indicating that TNF-α does not signal through a pertussis toxin–sensitive G protein in CVCs (Figure 2A). Instead, pertussis toxin induced ALP activity synergistically with TNF-α (Figure 2A). By 1-way ANOVA, the difference between control and TNF-α plus pertussis toxin (100 ng/mL) was significant (P<0.05 level; LSD 83.8). We previously showed that treatment with pertussis toxin alone (without subsequent TNF-α treatment) had relatively little effect on ALP activity.23

To determine whether TNF-α activated phospholipase A₂ (PLA₂) and cyclooxygenase, as found in bone cells,38 CVCs were pretreated with inhibitors of cytoplasmic PLA₂ (AA-
COCF3) or cyclooxygenase (indomethacin) for 2 hours before addition of TNF-α. ALP activity (measured 2 days after treatment) showed that these agents also did not inhibit TNF-induced ALP activity (Figure 2B). Instead, the ALP activity induced by TNF-α was further enhanced by the inhibitors. By 1-way ANOVA, the difference between control and each higher-dose treatment was significant (P<0.05 level; LSD 44.7). Treatments with inhibitor (AACOCF3 or indomethacin) alone had relatively little or no effect on ALP activity, CVC morphology, and total protein level (data not shown).

We also examined the role of the protein kinase C (PKC) pathway in TNF-α effects in CVCs. Inhibition of the PKC pathway by pretreatment with a PKC-specific inhibitor, chelerythrine chloride (0.5 to 1.0 μmol/L), for 2 hours did not reverse the TNF-α–induced ALP activity (Figure 2C). Instead, as seen with pertussis toxin and cyclooxygenase inhibitors, ALP activity induction by TNF-α was further enhanced by the chelerythrine chloride. By 1-way ANOVA, the difference between chelerythrine chloride–treated CVCs and the controls was significant (P<0.05; LSD 65.6). The same response was observed when PKC was downregulated by prolonged treatment (24 hours) with 1 mmol/L PMA (data not shown). Treatment of CVCs with chelerythrine chloride or PMA alone without subsequent TNF-α treatment had no effects on ALP activity or cell morphology (data not shown).

Because we have previously shown that the cAMP pathway promotes osteoblastic differentiation of CVCs,23 we also examined the involvement of the cAMP/protein kinase A (PKA) pathway in response to TNF-α. CVCs were treated with a PKA-specific inhibitor, KT5720.39 Treatment of CVCs for 2 hours with KT5720 before addition of TNF-α dose dependently inhibited the TNF-α–induced morphological change (Figure 3A). KT5720 also inhibited TNF-α–induced ALP activity (measured 2 days after treatment) (Figure 3B) and mineralization (measured 9 days after treatment) (Figure 3C) in a dose-dependent manner. Consistently, intracellular cAMP level was increased 2-fold in TNF-α–treated cells (Figure 3D).

**Effect of TNF-α on Transcription Factors Cbfa1/Osf2, AP1, and CREB**

Because TNF-α modulated the gene expression in CVCs as shown above, we also examined the effects of TNF-α on transcription factors important in osteoblastic differentiation: Cbfa1/Osf2, AP1, and CREB.36,40,41 Nuclear extracts from CVCs treated with TNF-α were prepared, and an electrophoretic mobility shift assay was performed with probes.

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**Figure 4.** Effect of TNF-α on DNA binding of transcription factors Cbfa1/Osf2, AP1, and CREB. DNA binding was analyzed by gel retardation assay. A, left panel, Nuclear extracts prepared from 2 separate samples treated for 3 days with either vehicle alone (control), forskolin (25 μmol/L), or TNF-α (10 ng/mL) for 3 days were incubated with 32P-labeled probe containing wild-type Cbfa1 binding probe.32 Bracket indicates mobility shift (n=4). A, right panel, Nuclear extracts from TNF-α–treated cells were preincubated with 2 μL of anti-Cbfa1/Osf2 antibody for 30 minutes before addition of labeled Cbfa1 binding probe (n=2).

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Arrow indicates supershifted complex. B, Nuclear extracts from CVCs treated with vehicle alone or TNF-α (10 ng/mL) for 3 days were incubated with AP1 binding oligo probe (n=3). A 100-fold excess of cold oligo probe was preincubated with nuclear extracts (third lane, n=2). C, left panel, Nuclear extracts from CVCs treated with TNF-α (10 ng/mL) for 3 days were incubated with CREB binding oligo probe (n=3). A 100-fold excess of cold oligo probe was preincubated with nuclear extracts (third lane, n=2). C, right panel, Nuclear extracts from TNF-α–treated cells were preincubated with 1 μL of anti-CREB antibody for 40 minutes before addition of labeled CREB binding probe. Arrow indicates supershifted complex.
containing Cbfa1/Osf2, AP1, or CREB binding sites. Results showed that treatment of CVCs with forskolin or TNF-α enhanced the DNA binding of Cbfa1/Osf2 (Figure 4A, left). Dibutyryl cAMP also enhanced DNA binding of Cbfa1/Osf2. The same concentration of dibutyryl cGMP had little or no effect (data not shown). Preincubation of anti-Cbfa1 antibody33 with TNF-α–treated nuclear extracts before addition of labeled probe resulted in a supershifted band and a decrease in the faster-migrating complex, indicating the specificity of Cbfa1/Osf2 (Figure 4A, right). TNF-α also enhanced DNA binding of AP1 and CREB transcription factors (Figure 4B and 4C). AP1 DNA binding was competed with 100-fold excess of cold AP1 oligo probe (Figure 4B), and CREB DNA binding was competed with 100-fold excess of cold CREB oligo probe (Figure 4C, left). Incubation of nuclear extracts from TNF-α–treated cells with anti-CREB antibody resulted in a supershifted complex (Figure 4C, right). Incubation with anti-Fos or anti-Jun antibodies, however, did not result in a supershifted complex, indicating that AP1 family members other than c-fos and c-jun were activated in response to TNF-α in CVCs (data not shown).

**Discussion**

The present study showed that TNF-α enhanced in vitro calcification of vascular cells, providing further evidence that vascular and bone calcification share regulatory factors. TNF-α facilitates CVC mineralization by increased expression and activity of ALP, an enzyme that has been shown to be important for matrix mineralization.36,42 The enhanced ALP may be a result of enhanced DNA binding of transcription factors shown to play a role in osteoblastic differentiation.36,40,41 These data, together with the previous report associating TNF-α with calcified vascular lesions in mice,29 raise the possibility that TNF-α facilitates mineralization in atherosclerotic lesions.

TNF-α has been shown to signal through different pathways in different cell types. In hepatic stellate cells and in osteoblasts, the TNF-α receptor is coupled to a pertussis toxin–sensitive G protein, leading to activation of phospholipase A2 and de novo synthesis of cyclooxygenase.6,37,38 In cultured rat mesangial cells and human fibroblasts, TNF-α signals through increased intracellular cAMP.43 Our results suggest that TNF-α triggers CVC mineralization via the cAMP pathway, based on the findings that PKA inhibition blocks and cAMP elevation mimics23 the TNF-induced effects. In addition, the finding that pertussis toxin synergizes with TNF-α in ALP induction further supports the possibility that TNF-α signals via the cAMP pathway in CVCs, because inhibition of Gαi by pertussis toxin enhances adenylate cyclase activity.

The results also suggested a form of cross talk between signaling pathways in CVCs. Inhibition of the PKC pathway potentiated the TNF-α effect on ALP activity, which suggests that the PKC pathway may act as an antagonist of PKA-mediated CVC differentiation. This may be due to involvement of PKC in cellular proliferation.44,45 The decline of which has been shown to be necessary for the onset of osteoblastic differentiation.36 The results also showed that similar effects were seen with inhibitors of the cyclooxygenase pathway, because activation of the PKC pathway by products of the cyclooxygenase pathway has been reported.46 Although CVCs share features with bone-derived osteoblasts in many aspects, we previously showed that their responses to certain agents such as oxidized lipids27 and active vitamin D are directly opposite.47 We and other investigators11–14 have found that TNF-α inhibited bone cell differentiation (unpublished data). The present results suggest that TNF-α has similar reciprocal effects (stimulation of CVCs and inhibition of bone cell differentiation). The coexistence of vascular calcification and osteoporosis has been reported recently by Bucay et al31 in mice lacking the osteoprotegerin gene, a novel soluble member of the TNF-α receptor superfamily. These inverse effects on bone and vasculature may be a result of different signaling pathways induced by TNF-α in the 2 cell types: stimulation of the cAMP pathway in CVCs versus stimulation of the pertussis toxin–sensitive G-protein–mediated pathway but not the cAMP pathway in osteoblasts.34 These differential responses to the same inflammatory agent may contribute in part to the paradoxical coexistence of vascular calcification and osteoporosis in older patients.

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**References**


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