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Activation of Caspases Is Required for Osteoblastic Differentiation*

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Previous studies have shown that mouse osteoblastic MC3T3-E1 cells undergo apoptosis when exposed to a mixture of proinflammatory cytokines. Bone morphogenetic protein (BMP)s are important regulators of osteoblast differentiation. Because regulation of osteoblastic differentiation is poorly understood, we sought to determine if BMP-4-induced differentiation of osteoblastic cells depends on the activity of the key apoptotic proteases, *i.e.* the caspases. BMP-4 induced the growth arrest and differentiation of osteoblastic cell line MC3T3-E1, as evidenced by the appearance of osteoblastic phenotypes such as alkaline phosphatase (ALP) activation and parathyroid hormone (PTH)-dependent production of cAMP. Surprisingly, BMP-4 induced transient and potent activation of caspase-8, caspase-2, and caspase-3, in this order. However, no apoptosis or necrosis in BMP-4-treated cells could be detected by FACS using annexin-V/propodium iodine double staining. Peptide inhibition of caspase activity led to a dramatic reduction in ALP activation and PTH-induced production of cAMP in BMP-4-treated cells. Although BMP-4 treatment resulted in cell-cycle G_0/G_1 arrest as detected by FACS cell-cycle analysis, caspase inhibitors (caspase-8, caspase-2, and caspase-3 inhibitors) could block the G_0/G_1 arrest in MC3T3-E1 cells. Taken together, these results confirm a unique and unanticipated role for the caspase-mediated signal cascade in the differentiation of osteoblasts.

Normal cell death is an important physiological process in which unnecessary cells are eliminated both during development and in adulthood (1). The processes involved in apoptosis are tightly regulated, and alterations in their function may result in disorders, including autoimmune disease and cancer (2). It is likely that osteoblasts may undergo programmed cell death because of their increased sensitivity to apoptosis-inducing agents in a special environment, *e.g.* excess cytokine exposure in inflammation sites (3). Therefore, apoptosis, especially in osteoblasts, may also play an important part in the duration and pathophysiology of bone-destructive diseases such as rheumatoid arthritis and periodontal diseases (4, 5). Apoptosis occurs as a consequence of the triggering of several complex signal cascades, and several lines of evidence indicate that caspases play an important role in apoptosis (6). First, caspase activation correlates with the onset of apoptosis, and caspase inhibition attenuates apoptosis (7). Second, *Caenorhabditis elegans* mutants lacking the worm caspase CED-3, have a complete absence of developmentally programmed cell death (8). More recently, targeted deletion of caspase genes has shown a definitive role for caspases in apoptosis and inflammation (9). Based on their structure and their ordering in cell death pathways, caspases have been classified into initiator and effector enzymes (10). Schematically, effector caspases, which include caspase-3, cleave a variety of cellular substrates; whereas initiator caspases such as caspase-2, -8, and -9 control the activation of the former enzymes (10). However, little is known as to the details of the signal cascade leading to caspase activation in osteoblastic cells, and alternative roles of caspase activation remain to be explored.

The cellular alterations associated with cell differentiation share a high degree of similarity with key phenotypic changes usually ascribed to apoptosis, suggesting that the induction of differentiation and apoptosis in cells may use overlapping cellular mechanisms (11). Recently, caspase activation, especially that of caspase-3, was shown to be required for erythroid and skeletal muscle differentiation (12, 13). Therefore, on the basis of the demonstrated signal efficacy of the death effector protease family, we investigated whether caspases may play a role during osteoblast differentiation. We report herein that the early part of the osteoblast differentiation program relies on the activity of 3 key apoptotic serine proteases, *i.e.* caspase-2, caspase-3, and caspase-8. Inhibition of the activity of various caspases severely attenuated alkaline phosphatase (ALP)[1] activation and parathyroid hormone (PTH)-induced production of cAMP, both of which are osteoblastic phenotypes. These results imply proapoptotic death effectors, previously thought to function in apoptosis and certain other events also have decisive context-dependent roles in the differentiation of osteoblasts.

EXPERIMENTAL PROCEDURES

Materials—7-Amino-4-trifluoromethyl coumarin (AFC), 7-amino-4-methyl-coumarin (AMC), Ac-YVAD-MCA as a caspase-1-like proteinase substrate, Ac-VDVAD-MCA as a caspase-2-like proteinase substrate, and Ac-DEVD-MCA as a caspase-3-like proteinase substrate were purchased from Peptide Institute (Osaka, Japan). Caspase-8-like proteinase substrate (Ac-IETD-AFC) was from Sigma; and caspase-9-like proteinase substrate (Ac-LEHD-AMC), from Bachem. Caspase-2-like protease inhibitor (Ac-VDVAD-CHO) and caspase-3-like protease inhibitor (Ac-DEVD-CHO) were purchased from Enzyme System Prod. A caspase-8-like protease inhibitor (Ac-IETD-CHO) and caspase-9-like protease inhibitor (Ac-LEHD-CHO) were from Biomol Res. Laboratory. These peptide inhibitors have been used previously to analyze the possible role of these 4 caspases in the intracellular process of apoptosis

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[1] The abbreviations used are: ALP, alkaline phosphatase; AFC, 7-amino-4-trifluoromethyl coumarin; AMC, 7-amino-4-methylcoumarin; α-MEM, α-minimal essential medium; BMP, bone morphogenetic protein; BrdUrd, 5-bromo-2′-deoxyuridine; FACS, fluorescence-activated cell sorter; mAb, monoclonal antibody; PI, propidium iodine; PTH, parathyroid hormone; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay.

in various mammalian cells *in vitro*. Recombinant human bone morphogenetic protein (BMP)-4 was purchased from R & D Systems (Minneapolis, MN). The antibody against caspase-1 was obtained from Medical Biological Lab (Nagoya, Japan); and that against caspase-9, from Cell Signaling Tech. The antibodies against caspase-3 and caspase-8 were from BD Biosciences.

Cell Culture—MC3T3-E1 cells, established from newborn mouse calvaria, are a clonal osteogenic cell line whose cells can differentiate into osteoblasts. These cells were cultured in plastic dishes containing α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in air with 5% CO_2, and were then subcultured until they had become semiconfluent.

Cell Proliferation—Cell proliferation assay was performed by the detection of 5-bromo-2′-deoxyuridine (BrdUrd)-labeled DNA by use of anti-BrdUrd monoclonal antibody (mAb) labeled with peroxidase (Cell Proliferation ELISA, Roche Applied Science). In short, MC3T3-E1 cells grown in a 96-well microtiter plate (3×10^3 cells/well) were incubated for different periods of time at 37 °C in a humidified atmosphere (5% CO_2) in the presence of BMP-4 (10 ng/ml), and were then labeled with BrdUrd for 1 h. The amount of BrdUrd-labeled DNA was quantified by the ELISA.

Assessment of the Osteoblastic Phenotype—To assess the phenotype of the cultured cells, we examined them for ALP activity and responsiveness to PTH. ALP activity was determined by use of Blue-Phos substrate (microwell phosphatase substrate system: Kirkegaard & Perry Laboratory). The response to PTH was determined by measuring the amount of cAMP produced by cells with or without exposure to 200 ng/ml human PTH (hPTH (1–34) Peptide Institute Inc.) for 15 min. The concentration of cAMP in cells was measured by an ELISA (Cayman Co.).

Determination of Apoptotic Cell Death and Cell Cycle Analysis with a Fluorescence-activated Cell Sorter (FACS)—Apoptosis was evaluated with a FACS Calibur (BD Biosciences), using the annexin-V-FITC & propidium iodine (PI) double staining method (apoptosis detection kit, MBL). Cell cycle analysis was also evaluated with the FACS using PI and a cell cycle analysis kit (Cycle Test plus, BD Biosciences) according to the manufacturer's protocol.

Determination of Apoptotic Cell Death by ELISA—Cellular DNA fragmentation assay was performed by the detection of BrdUrd-labeled DNA fragments in the cytoplasm of cell lysates by use of solid phase-immobilized anti-DNA mAb and anti-BrdUrd mAb labeled with peroxidase (cellular DNA fragmentation ELISA, Roche Applied Science). In short, MC3T3-E1 cells grown in culture dishes (1×10^5 cells/ml) were labeled with BrdUrd for 15 h; and the cell concentration was then adjusted to 5×10^4 cell/ml, and 100 μl of suspension was transferred to each well of a microtiter plate (Nunc, Denmark, 96-well, flat bottom). Cells were then incubated in the presence of BMP-4 for 24 or 48 h at 37 °C in a humidified atmosphere (5% CO_2). The treatment with TNF-α was used as a positive control. The amount of BrdUrd-labeled DNA released into the cytoplasm of apoptotic cells was quantified by ELISA.

Assay for Caspase-1, Caspase-2, Caspase-3, Caspase-8, and Caspase-9—MC3T3-E1 cells in 100-mm dishes were lysed with 400 μl of lysis buffer (10 mM HEPES, pH 7.5, containing 0.5% Nonidet P-40, 0.5 mM EDTA, 150 mM NaCl, and 2 mM phenylmethylsulfonyl fluoride). Aliquots (100 μl) of the extracts were incubated for 1 h at 37 °C with 50 μM enzyme substrate (Ac-YVAD-MCA for caspase-1-like proteinase, Ac-VDVAD-MCA for caspase-2-like protease, Ac-DEVD-MCA for caspase-3-like proteinase, Ac-IETD-AFC for caspase-8-like proteinase, and Ac-LEHD-MCA for caspase-9-like proteinase) in 10 mM HEPES, pH 7.5, containing 0.05 M NaCl, and 2.5 mM dithiothreitol in a 300-μl reaction mixture. The fluorescence of the released AMC was measured with a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. For the caspase-8 assay, the fluorimeter was calibrated by using known concentrations of free AFC (excitation = 400 nm, emission = 505 nm) to generate a standard curve of fluorescence *versus* μmol of AFC. For specific inhibition of caspase activity, cells were pretreated with a caspase inhibitor for 1 h at 37 °C, and then the caspase assay was performed. Protein concentration of the extract was estimated by conducting a DC-Bio-Rad Protein assay using bovine serum albumin as a standard.

Western Blot Analysis of Caspase Processing—Western blotting of caspase processing was performed by the method of Chua *et al.* (14) with slight modification. MC3T3-E1 cells were lysed in caspase lysis buffer, and centrifuged at $14{,}000 \times g$ for 10 min to remove insoluble materials. Lysed protein content was determined with bovine serum albumin employed as a standard, by means of a DC-Bio-Rad protein assay kit. Lysates were dissolved in SDS electrophoresis buffer; and proteins were separated on SDS-polyacrylamide gels (12%) and subsequently electrotransferred to polyvinylidene difluoride membranes. After having been blocked with phosphate-buffered saline containing 5% bovine serum albumin, the membranes were incubated overnight at 4 °C with an antibody to either caspase-1, caspase-3, caspase-8, or caspase-9; and the bound antibodies were detected with the corresponding secondary antibody conjugated to alkaline phosphatase. Blots were developed by fluorescence-releasing substrate from Amersham Biosciences. Visualization of the bands in the membranes was performed with a FluorImager (Molecular Dynamics).

Statistical Analysis—Data were presented as the mean ± S.E. of 4–6 cultures/group. Each experiment was repeated three times. Differences between control and experimental treatment groups were determined by using the paired Student's t test. Differences were considered significant if $p < 0.05$.

RESULTS

BMP-4 induces mouse bone marrow stromal cells, *e.g.* ST-2, MC3T3-G2/PA6, and MC3T3-E1 cells, to differentiate and express osteoblastic phenotypes such as ALP activation and PTH-dependent production of cAMP (15). To determine whether caspases could play a role in BMP-4-induced osteoblastic differentiation, we utilized mouse osteoblastic MC3T3-E1 cells treated with human recombinant active BMP-4. We found that BMP-4 (10 ng/ml) caused a profound and transient activation of caspase-2, caspase-3, and caspase-8, with a maximum effect at 90 min (Fig. 1*A*). However, BMP-4 did not activate caspase-1 and caspase-9 in cells. Western blot analysis of caspase processing also demonstrated that BMP-4 action resulted in a time-dependent decrease in pro-caspase-3 (32 kDa) and caspase-8 (55 kDa), with a maximum effect at 90 min (Fig. 1*B*). In addition, we detected the band for active caspase-3 (18 kDa) in BMP-4 treated cells. Procaspase-1 and procaspase-9 remained at a constant level through out the experiment. Unfortunately, since commercially available antibodies for caspase-2 could not detect any band of procaspase-2 or processed peptide, we have no Western blot data on caspase-2 processing. Dose-dependent examination showed that BMP-4 treatment resulted in caspase activation up to the maximum at 30 ng/ml.

Using several specific caspase inhibitors, we examined the order of caspase activation in BMP-4 treated cells (Fig. 2). BMP-4-induced activation of caspase-8 was inhibited by the caspase-8 peptide inhibitor (Fig. 2*A*). Although BMP-4- induced activation of caspase-2 was inhibited by caspase-2 and caspase-8 inhibitor, respectively, the activation of caspase-3 was inhibited by all caspase inhibitors tested (Fig. 2*A*). Western blot analysis also confirmed that all caspase inhibitors tested attenuated the level of BMP-4-induced active caspase-3 (18 kDa, Fig. 2*B*). Western blot analysis of caspase-8 processing yielded results similar to those shown in Fig. 2*A*. Taken together, we demonstrated that BMP-4 caused transient caspase-8, caspase-2-like proteinase, and caspase-3 activation to occur, in this order.

Activation of the caspase cascade usually leads to DNA degradation, externalization of phosphatidylserine on the plasma membrane, and apoptotic cell death (6). Conceivably, the activation of caspases may be required to remove a population of osteoblasts (through apoptosis) that would normally inhibit the differentiation process. Alternatively, elevated caspase activities may lead to intracellular alterations, which, in turn, activate a differentiation program. To determine whether BMP-4 could trigger apoptotic cell death, we monitored cell proliferation and DNA fragmentation ELISA, and conducted FACS cell-cycle analysis and FACS annexin-V/PI double staining analysis. BMP-4 treatment resulted in the potent growth arrest, which occurred in a dose-dependent manner (Fig. 3*A*). However, BMP-4 could not induce DNA fragmentation up to 48 h; whereas TNF-α as a positive control could do so (Fig. 3*B*). Although hypodiploid DNA peaks are commonly found in the

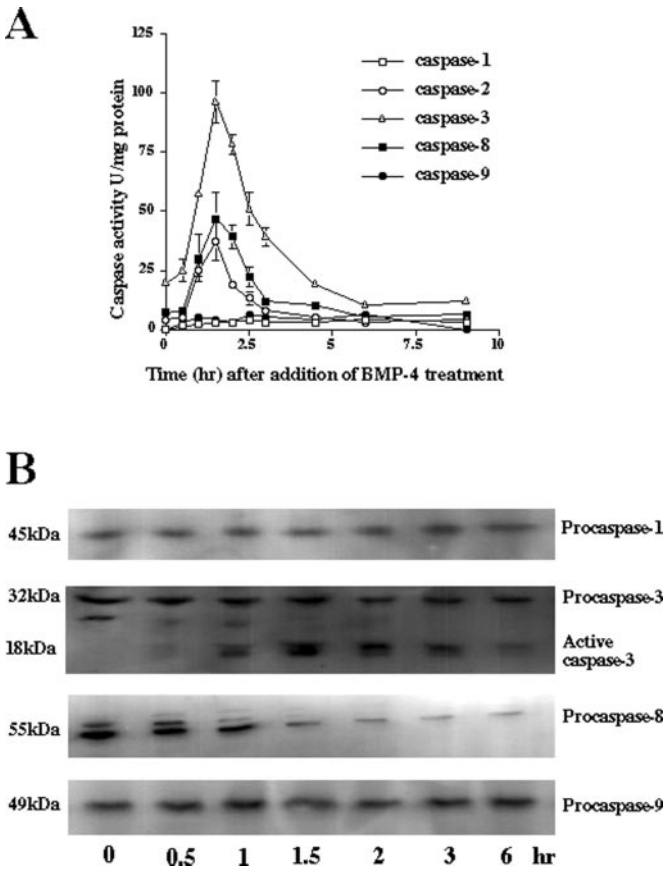

FIG. 1. *A*, time-dependent activation of caspase-2, -3, and -8 in BMP-4-treated MC3T3-E1 cells. MC3T3-E1 cells were plated in 100-mm-diameter dishes (1×10^5 cells) and cultured for the indicated times in the presence of BMP-4 (10 ng/ml). The activity of various caspases in the cell lysates was assayed by the standard method. MC3T3-E1 cells were lysed with 400 μl of lysis buffer, and the extracts were incubated with 50 μM enzyme substrate (Ac-YVAD-MCA for caspase-1-like proteinase, Ac-VDVAD-MCA for caspase-2 like proteinase, Ac-DEVD-MCA for caspase-3-like proteinase, Ac-IETD-AFC for caspase-8-like proteinase, and Ac-LEHD-MCA for caspase-9-like proteinase) for 1 h at 37 °C. One unit was defined as the amount of the enzyme required to released 1 μmol of AMC per hour at 37 °C. Protein concentration of the extract was estimated by the DC-Bio-Rad Protein assay using bovine serum albumin as a standard. Data are expressed as the mean ± S.E. of 4 samples. *B*, Western blot analysis of caspase processing. Western blotting was performed by the method of Chua *et al.* (14) with slight modification. Protein content of lysates was determined with bovine serum albumin employed as a standard by means of a DC-Bio-Rad protein assay kit. Equal amounts of total protein were applied for electrophoresis. The blotted membranes (polyvinylidene difluoride membranes) were incubated overnight at 4 °C with an antibody to either caspase-1, caspase-3, caspase-8, or caspase-9; and the bound antibodies were detected with the secondary antibody conjugated to alkaline phosphatase. Blots were developed by fluorescence-releasing substrate. Visualization of the bands in the membranes was performed with a FluorImager.

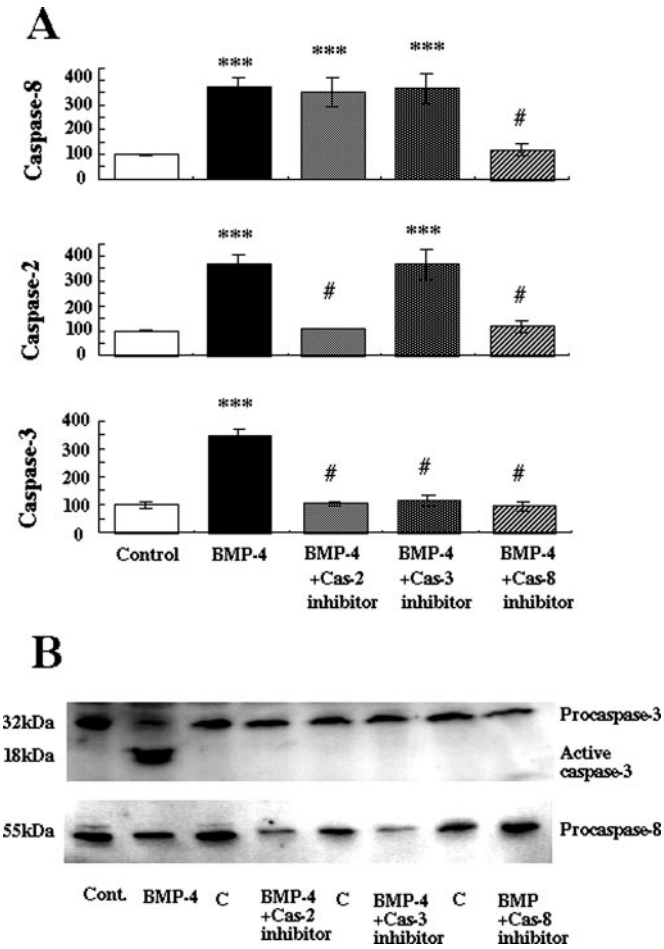

FIG. 2. *A*, activation of caspase-8, -2, and -3 in MC3T3-E1 cells treated with the combination of BMP-4 and a given caspase inhibitor. For specific inhibition of caspase activity, cells were pretreated with a caspase inhibitor (each concentration: 3 μM) for 1 h at 37 °C and then incubated for 1.5 h in the presence of BMP-4 (10 ng/ml) and the caspase inhibitor. Then, the caspase assay was performed. Each experiment was repeated three times, and data shown are representative of three independent experiments. Data are expressed as the mean ± S.E. of four samples. ***, $p < 0.005$ (compared with control); #, <0.01 (compared with BMP-4-treated cells). *Cas-2 inhib.*, caspase-2 inhibitor; *Cas-3 inhib.*, caspase-3 inhibitor; *Cas-8 inhib.*, caspase-8 inhibitor; *Cas-9 inhib.*, caspase-9 inhibitor. *B*, Western blot analysis of caspase-3 and -8 processing. For specific inhibition of caspase activity, cells were pretreated with a caspase inhibitor (each concentration: 3 μM) for 1 h at 37 °C and then incubated for 1.5 h in the presence of BMP-4 (10 ng/ml) and the caspase inhibitor. Then, Western blot analysis was performed.

case of apoptosis, BMP-4 did not produce a significant increase in the proportion of hypodiploid cells up to 48 h (data not shown). Surprisingly, no measurable differences were found in the stage-marker of apoptosis and/or necrosis, as demonstrated by flow cytometry with annexin V and PI (Fig. 3*C*). These results indicate that BMP-4 induced growth arrest in MC3T3-E1 cells but that elevated caspase activities in cells were not associated with apoptotic cell death.

Although we have little insight into the reason for the BMP-4-induced growth arrest in osteoblastic cells, we found that BMP-4 caused potent G_0/G_1 arrest in MC3T3-E1 cells as early as 24 h after the start of BMP-4 treatment (Fig. 4). The cell cycle arrest was pronounced, with almost 85% of cells in the G_0/G_1 stage as opposed to 67% in the control cultures; and very few cells could be found in either the S (control *versus* BMP-4: 28.3 *versus* 12.3%) or the G_2/M (5.4 *versus* 2.7%) phases, with nearly all cells having been arrested in G_0/G_1 ($p < 0.005$ for control *versus* BMP-4: Table I). We next investigated whether the caspases (caspase-8, caspase-2, and caspase-3) played a mediating role in the BMP-4-induced G_0/G_1 arrest in cells. Surprisingly, pretreatment of cells with the caspase-2, caspase-3, or caspase-8 inhibitor could prevent the G_0/G_1 arrest in the BMP-4-treated cells (Table I). We confirmed that the caspases tested were not activated in the presence of their respective inhibitor (data not shown).

To assess osteoblast differentiation, we measured ALP activity and PTH-responsive cAMP production in the MC3T3-E1 cells. The control MC3T3-E1 cells had very low levels of ALP activity and a low response to PTH in terms of producing cAMP (Fig. 5, *A* and *B*). Exposure of cells to BMP-4 for 2 days significantly increased the ALP activity up to 4-fold of the control, and also induced responsiveness for PTH-elicited cAMP pro-

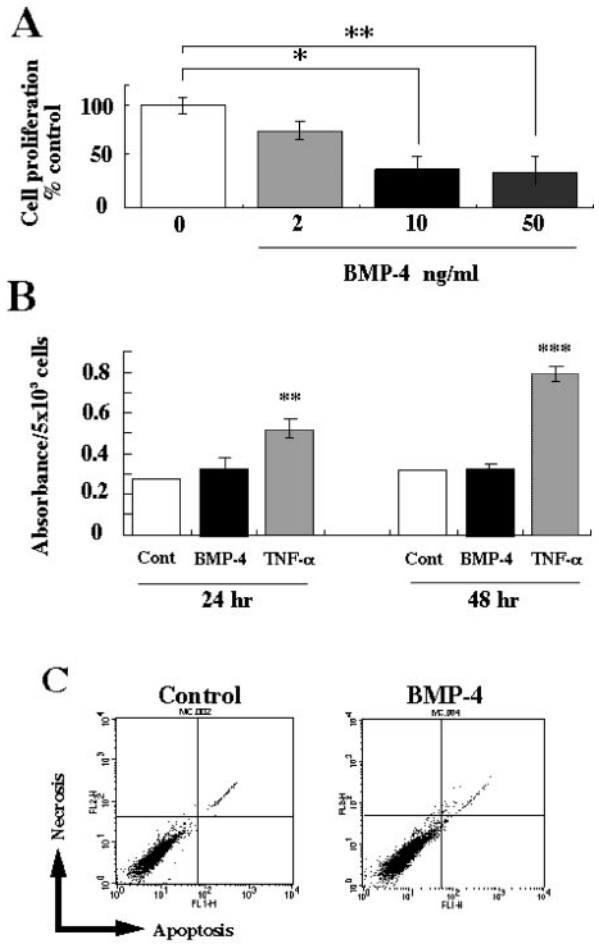

FIG. 3. *A*, cell proliferation of MC3T3-E1 treated with BMP-4. Cells were incubated for 24 h in the presence of BMP-4 (0, 2, 10, or 50 ng/ml) in 10% fetal calf serum in α-MEM. Cells were then incubated with BrdUrd for 1 h, and the incorporated BrdUrd was detected with anti-BrdUrd antibody labeled with peroxidase (BrdUrd proliferation ELISA kit: Roche Applied Science). Data are expressed as the mean ± S.E. of six samples. *, $p < 0.05$; **, $p < 0.01$ (compared with control). *B*, quantitative analysis of DNA fragmentation. MC3T3-E1 cells in 96-well dishes were treated with BMP-4 (10 ng/ml) or TNF-α (20 ng/ml) as a positive control for 24 h or 48 h. Cell lysates were subjected to ELISA for DNA fragmentation. Data are expressed as the mean ± S.E. of six samples. **, $p < 0.01$; ***, $p < 0.005$ (compared with control). *C*, annexin-V-FITC/PI double staining of BMP-4-treated MC3T3-E1 cells. Cells were incubated for 24 h in the presence of BMP-4 (10 ng/ml). Cells were then incubated with annexin-V-FITC and PI (annexin V-FITC apoptosis detection kit: Medical & Biological Lab., Nagoya, Japan), after which the exposure of phosphatidylserine and PI-positive cells in MC3T3-E1 cells were evaluated with the FACS calibur (Software: CellQuest). Each experiment was repeated three times, and data shown are representative of three independent experiments.

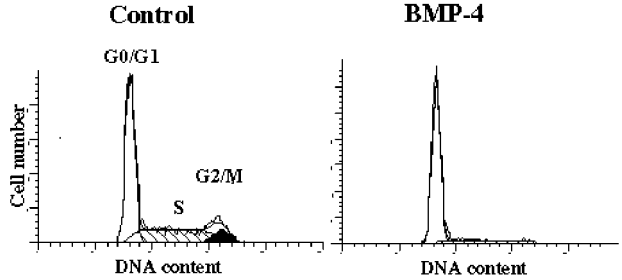

FIG. 4. **Cell-cycle analysis of MC3T3-E1 cells treated with BMP-4.** Cells were incubated for 24 h in the presence of BMP-4 (10 ng/ml). Cells were first incubated with PI (Cycle test Plus, DNA reagent kit: BD Biosciences), and then PI-DNA in MC3T3-E1 cells was evaluated with the FACS calibur (Software: ModFit). Each experiment was repeated three times, and data shown are representative of three independent experiments.

duction with statistical significance ($p < 0.005$). However, pretreatment with the caspase inhibitors (caspase-2, -3, and -8) substantially reduced both responses in cells (Fig. 5), thus indicating that the elevated caspase activities were associated with differentiation of MC3T3-E1 cells into phenotypic mouse osteoblastic cells.

DISCUSSION

Our present results point to a novel role for caspases in cell differentiation. Treatment of MC3T3-E1 cells with BMP-4 resulted in potent growth arrest but not apoptosis, as well as in a profound and transient activation of caspase-8, caspase-2-like proteinase, and caspase-3 (Fig. 1). We also obtained similar results showing that BMP-4 treatment resulted in the potent activation of caspase-2-like proteinase, caspase-3, and caspase-8, and that the pretreatment with caspase inhibitors could attenuate BMP-4-induced ALP activation and PTH-response in ST-2 mouse stromal cells (data not shown), suggesting that this phenomenon is not limited to mouse osteoblastic MC3T3-E1 cells. BMP-4 regulates the expression of mouse tissue transglutaminase (16). Although a previous study demonstrated that BMP-2 could induce the activation of caspases and apoptosis in osteoblasts (17), we found that BMP-4 treatment of MC3T3-E1 cells did not lead to apoptosis but to differentiation.

When a cell moves into a quiescent and/or terminally differentiated state from G_1, it clearly enters a different metabolic state. This state (G_0) has not yet been given a precise molecular, biochemical, or genetic definition. In general, G_0/G_1 arrest in the cell cycle is induced by apoptotic stimulators and/or occurs in cells in the terminally differentiated state. BMP is known to induce the differentiation of osteoblastic cells having specific phenotypes such as ALP activation, PTH-dependent production of cAMP, and expression of osteocalcin and CBFA1 (15, 18, 19). Since we confirmed that BMP-4 treatment resulted in potent growth arrest and G_0/G_1 arrest in MC3T3-E1 cells (Figs. 3*A* and 4) and increased ALP activity and PTH-dependent production of cAMP (Fig. 5), these cells likely had begun differentiation. Surprisingly, pretreatment of cells with caspase inhibitors could attenuate the G_0/G_1 arrest in the BMP-4 treated cells (Table I), and substantially reduced the responses for BMP-4-induced ALP activation and the response to PTH by producing cAMP in cells (Fig. 5). Although caspases, a specific group of cysteine proteases, have been found to play an essential role in apoptosis by cleaving important cellular proteins (6), the presently found activation of these proteases did not lead to apoptotic and/or necrotic cell death (Fig. 3). Taken together, our observations from this cell culture model support the hypothesis that activation of endogenous caspases is required for osteoblast differentiation but does not lead to apoptotic cell death.

Although a recent study on BMP-induced signal cascades focused on SMAD family molecules (20), we demonstrated that activated caspases are involved in BMP-induced signal cascades. Especially, caspase-8, central, and a long predomain-containing caspase, is located at the apex of the cell death receptor-mediated apoptotic cascade. Overall, this signal cascade starts with the activation of caspase-8, the active form of which leads to the sequential activation of caspase-3 and related cysteine proteases, resulting in the proteolytic cleavage of various cellular substrates; and such cleavages, in turn, lead to the characteristic morphologic and biochemical changes that occur during apoptosis (21). In accordance with previous findings, we confirmed that BMP-4 caused transient activation of

TABLE I
Effect of caspase inhibitors on the BMP-4-induced G_0/G_1 arrest in MC3T3-E1 cells as studied by FACS cell-cycle analysis

	Cell cycle		
	G_0/G_1	S	G_2/M
		%	
Control	66.95 ± 3.71	28.30 ± 3.22	5.42 ± 1.81
BMP-4	85.01 ± 5.89***	12.26 ± 3.25***	2.73 ± 0.58
BMP-4 + caspase-2 inhibitor	66.41 ± 0.64	28.06 ± 1.38	5.56 ± 1.62
BMP-4 + caspase-3 inhibitor	67.37 ± 3.29	27.35 ± 2.60	5.20 ± 3.29
BMP-4 + caspase-8 inhibitor	66.87 ± 8.54	26.73 ± 5.22	6.40 ± 1.68
BMP-4 + caspase-9 inhibitor	85.31 ± 5.77***	12.76 ± 2.88***	1.86 ± 1.91

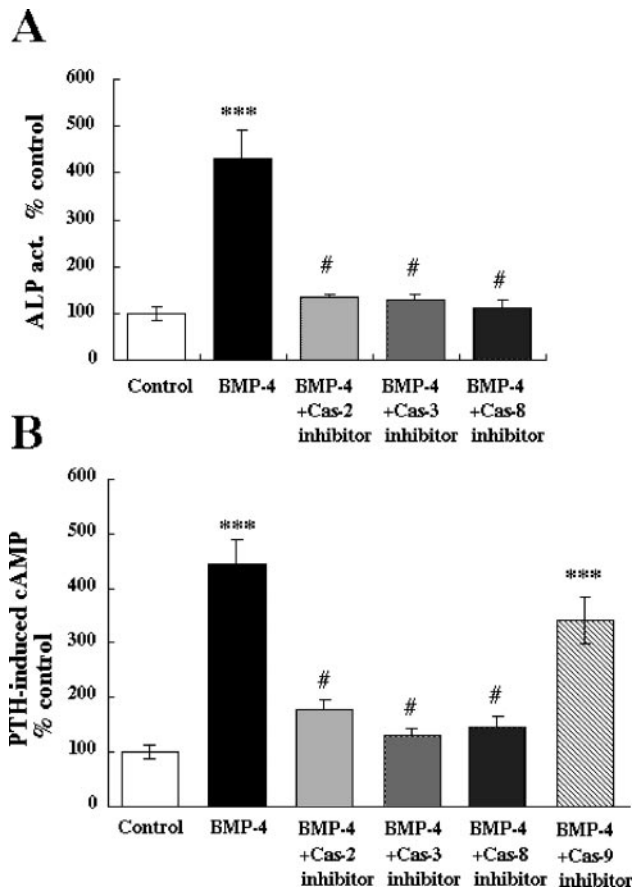

FIG. 5. *A*, inhibition of BMP-4-induced alkaline phosphatase (ALP) in MC3T3-E1 cells by caspase inhibitors. MC3T3-E1 cells were plated in 100-mm-diameter dishes (1×10^5 cells) and incubated for 48 h in the presence of BMP-4 (10 ng/ml) and a given caspase inhibitor (each concentration: 3 μM). Cells were lysed, and the extracts were incubated with Blue-Phos substrate for 1 h at 37 °C (microwell phosphatase substrate system: Kirkegaard & Perry Laboratory). Data are expressed as the mean ± S.E. of six samples. ***, $p < 0.005$ (compared with control); #, <0.01 (compared with BMP-4-treated cells). *B*, parathyroid hormone (PTH)-dependent production of cAMP in MC3T3-E1 cells treated with BMP-4 combined with a given caspase inhibitor. MC3T3-E1 cells were plated in 100-mm diameter dishes (1×10^5 cells) and incubated for 48 h in the presence of BMP-4 (10 ng/ml) and a given caspase inhibitor (each concentration: 3 μM). The response to PTH was determined by measuring the amount of cAMP produced by cells with or without exposure of 200 ng/ml human PTH (hPTH-(1–34)) for 15 min. The concentration of cAMP in cells was measured by an ELISA (Cayman Co.). Data are expressed as the mean ± S.E. of six samples. ***, $p < 0.005$ (compared with control); #, <0.01 (compared with BMP-4-treated cells).

caspase-8, caspase-2-like proteinase, and caspase-3 in this order (Figs. 1 and 2).

Furthermore, previous reports suggested that caspase-3 is capable of regulating nonapoptotic functions in certain cell types, *e.g.* nuclear extrusion in differentiating lens epithelium and keratinocytes, T cell activation, erythroid, and myoblasts (22, 23). Although the relation between caspase-3 and the cell cycle, especially with respect to G_0/G_1 arrest, has not been examined, caspase-3 activation was reported to be required for erythroid and skeletal muscle differentiation (12, 13). Active caspase-3 is known to be involved in caspase activation loop (24, 25). By using a protein transfection system, Fernando *et al.* (13) demonstrated that the activation of caspase-3 was required for skeletal muscle differentiation. To further test a cell autonomous role of caspase-3 in inducing osteoblast differentiation, we transfected a subconfluent population of MC3T3-E1 cells with recombinant active caspase-3 protein; however, we do not yet have any solid data by this approach (data not shown). Since we demonstrated the position of caspase-3 is relatively downstream in BMP-4-induced cascade in Fig. 2, the activation of caspase-3 may plays an important role in the differentiation of osteoblasts.

In addition to caspase-3 and caspase-8, caspase-2-like proteinase showed a novel involvement in osteoblastic cell differentiation. Our observations directly link caspase activities (caspase-8, caspase-2-like proteinase, and caspase-3) to such profound alterations of phenotypes in cell differentiation without ushering in a death-like response. Therefore, other activated caspases (*e.g.* downstream of caspase-3) might play an important role in the degradation of specific nuclear proteins and lead to osteoblastic differentiation.

Taken together, our data demonstrate that normal osteoblast differentiation requires the transient activation of several caspases. Although these proteases are known to be involved in several physiological processes, including apoptotic cell death, inflammation, and T cell proliferation (23, 24), our data indicate a novel physiological function of caspases. Further exploration of the role of caspases in osteoblastic cells would be of major interest for understanding the fate (differentiation *versus* apoptosis) of osteoblastic cells under different physiological or pathological conditions.

Addendum—After we obtained the results presented in this article, we became aware of concurrent research (Refs. 12 and 13) that addresses questions similar to ours, albeit through different methods. In preparing our manuscript, we referred to these articles and, in several instances, used sentences verbatim from them, basically because of our difficulty with expressions in English. However, we now realize the inappropriateness of such action and sincerely apologize to the authors of those published articles.

REFERENCES

1. Cohen, J. J. (1993) *Immunol. Today* **14,** 126–130
2. Thompson, C. B. (1995) *Science* **267,** 1456–1462
3. Jilka, R. L., Weinstein, R. S., Bellido, T., Parfitt, A. M., and Manolagas, S. C. (1998) *J. Bone Miner. Res.* **13,** 793–802
4. Mogi, M., Kinpara, K., Kondo, A., and Togari, A. (1999) *Biochem. Pharmacol.* **58,** 649–654
5. Morimoto, Y., Morimoto, H., Murata, T., Kobayashi, S., Ohba, T., and Haneji, T. (1999) *J. Dent. Res.* **78,** 735–742
6. Wolf, B. B., and Green, D. R. (1999) *J. Biol. Chem.* **274,** 20049–20052
7. Bump, N, J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferenz, C., Franklin, S., Ghayur, T., Li, P., Licari, P., Mankovich, J., Shi, L., Greenberg, A. H., Miller, L. K., and Wong, W. W. (1995) *Science* **269,** 1885–1888
8. Ellis, H. M., and Horvitz, H. R. (1986) *Cell* **44,** 817–829

9. Green, D. R. (1998) *Cell* **94,** 695–698
10. Nicholson, D. W., and Thornberry, N. A. (1997) *Trends. Biochem. Sci.* **22,** 299–306
11. Zamzami, N., and Kroemer, G. (1999) *Nature* **401,** 127–128
12. Zelmati, Y., Garrido, C., Amsellem, S., Fishelson, S., Bouscary, D., Velensi, F., Varet, B., Solony, E., and Hermine, O. (2001) *J. Exp. Med.* **193,** 247–254
13. Fernando, P., Kelly, J. F., Balazsi, K., Slack, R. S., and Megeney, L. A. (2002 *Proc. Natl. Acad. Sci. U. S. A.* **99,** 11025–11030
14. Chua, C. C., Chua, B. H., Chen, Z., Landy, C., and Hamdy, R. C. (2002) *Biochim. Biophys. Acta* **1593,** 1–8
15. Yamaguchi, A., Ishizuka, T., Kintou, N., Wada, Y., Katagiri, T., Wozney, J. M., Rosen, V., and Yoshiki, S. (1996) *Biochem. Biophys. Res. Commun.* **220,** 366–371
16. Ritter, S. J., and Davies, P. J. (1998) *J. Biol. Chem.* **273,** 12798–12806
17. Hay, E., Lemonnier, J., Fromigue, O., and Marie, P. J. (2001) *J. Biol. Chem.* **276,** 29028–29036
18. Yamaguchi, A., Katagiri, T., Ikeda, T., Wozney, J. M., Rosen, V., Wang, E. A., Kahn, A. J., Suda, T., and Yoshiki, S. (1991) *J. Cell Biol.* **113,** 681–687
19. Gori, F., Thomas, T., Hicok, K. C., Spelsberg, T. C., and Riggs, B. L. (1999) *J. Bone Miner. Res.* **14,** 1522–1535
20. Hoffmann, A., and Gross, G. (2001) *Crit. Rev. Eukaryot. Gene Expr.* **11,** 23–45
21. Nagata S. (1997) *Cell* **88,** 355–365
22. Ishizaki, Y., Jacobson, M. D., and Raff, M. C. (1998) *J. Cell Biol.* **140,** 153–158
23. Alam, A., Cohen, L. Y., Aouad, S., and Sekaly, R. P. (1999) *J. Exp. Med.* **190,** 1879–1890
24. Srinivasula, S. M., Fernandes-Alnemri, T., Zangrilli, J., Robertson, N., Armstrong, R. C., Wang, L., Trapani, J. A., Tomaselli, K. J., Litwack, G., and Alnemri, E. S. (1996) *J. Biol. Chem.* **271,** 27099–27106
25. Metkar, S. S., Wang, B., Ebbs, M. L., Kim, J. H., Lee, Y. J., Raja, S. M., and Froellich, C. J. (2003) *J. Cell Biol.* **160,** 875–885