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# 1α,25-Dihydroxyvitamin $D_3$ regulates the expression of carbonic anhydrase II in nonerythroid avian bone marrow cells

(myelomonocytes/osteoclasts/macrophages/bone resorption/acid–base regulation)

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**ABSTRACT** **1α,25-Dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$], the active metabolite of the steroid hormone vitamin D, is a potent regulator of macrophage and osteoclast differentiation. The mature osteoclast, unlike the circulating monocyte or the tissue macrophage, expresses high levels of carbonic anhydrase II (CAII). This enzyme generates protons and bicarbonate from water and carbon dioxide and is involved in bone resorption and acid–base regulation. To test whether 1,25$(OH)_2D_3$ could induce the differentiation of myelomonocytic precursors toward osteoclasts rather than macrophages, we analyzed its effects on the expression of CAII in bone marrow cultures containing precursors common to both cell types. The expression of CAII was markedly increased by 1,25$(OH)_2D_3$ in a dose- and time-dependent manner. In bone marrow, this increase occurred at the mRNA and protein levels and was detectable as early as 24 hr after stimulation. 1,25$(OH)_2D_3$ was also found to induce CAII expression in a transformed myelomonocytic avian cell line. These results suggest that 1,25$(OH)_2D_3$ regulates the level at which myelomonocytic precursors express CAII, an enzyme that is involved in the function of the mature osteoclast.**

1α,25-Dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$] is the active metabolite of the steroid hormone vitamin D (1). This metabolite regulates the differentiation of osteoclasts and macrophages, two cell types that are thought to be derived from a common myelomonocytic precursor (2–6). 1,25$(OH)_2D_3$ induces an increase in bone resorption *in vivo* (1, 7) and in organ cultures (8, 9) but has no effect on the resorptive activity of isolated osteoclasts (10, 11). The increase in bone resorption is believed to be mediated primarily by means of an effect upon the proliferation and/or differentiation of osteoclast precursors within the hematopoietic bone marrow (12–15), leading to an increase in the number of mature osteoclasts (7, 9). This view is supported by *in vitro* experiments with normal bone marrow cells or transformed cell lines. First, 1,25$(OH)_2D_3$ induces the differentiation and maturation of myelomonocytic cell lines toward the mononuclear phagocyte rather than the granulocyte lineage (3, 12, 15–18). Further, 1,25$(OH)_2D_3$ stimulates the formation of multinucleated osteoclast-like cells in long-term bone marrow cultures (13, 14, 19, 20) and increases the proportion of the cells that can resorb bone (14). Taken together, these results suggest that this steroid hormone may specifically induce the differentiation of myelomonocytic precursors toward the macrophage and/or osteoclast lineage.

One of the genes that is expressed at high levels in the osteoclast (21–23), but not in peripheral monocytes or mature macrophages (24), is the gene encoding carbonic anhydrase II (CAII). CAII is an enzyme that reversibly generates bicarbonate and protons from carbon dioxide and water (25). This enzyme plays an essential role in acid-secreting cells such as the kidney tubule intercalated cell (26), the gastric mucosa oxyntic cell (27), and the osteoclast (21). In humans, defective CAII is associated with cerebral calcification, tubular acidosis, and osteopetrosis (28), providing genetic evidence for CAII involvement in bone resorption. An analogue of the human disease has been produced by chemical mutagenesis in the mouse, where CAII deficiency is associated with renal tubular acidosis and vascular calcification but no major bone defects (29).

We have investigated whether CAII expression is regulated in bone marrow cultures by 1,25$(OH)_2D_3$. We find that the levels of expression of CAII mRNA and protein are markedly increased by 1,25$(OH)_2D_3$ during the *in vitro* differentiation of normal bone marrow precursors. This was also observed in a transformed myelomonocytic cell line (BM2), thereby confirming a recent report in the transformed human monocytic cell line HL60 (30). These results suggest that 1,25$(OH)_2D_3$ acts directly on myelomonocytic precursor cells to induce expression of CAII, a protein that is present at high levels in the fully differentiated osteoclast.

## MATERIAL AND METHODS

**Cell Preparations.** Bone marrow cells were isolated from the tibias and femurs of White Leghorn chickens (SPAFAS, Norwich, CT) fed a calcium-deficient diet (Purina) for 1–2 weeks after hatching. Cells passed through a 10-μm nylon filter (Small Parts) to remove mature multinucleated osteoclasts were fractionated on Ficoll-Paque (Pharmacia) and plated for 16 hr at $5 \times 10^6$ cells per ml in alpha minimum essential medium (Sigma) containing heat-inactivated chicken (2%) and fetal calf (8%) sera (Sigma), 50 units of penicillin per ml, and 50 μg of streptomycin per ml. To deplete these preparations of the most mature stromal and myelomonocytic cells (31), nonadherent cells were recovered after a 16-hr culture and replated at $10^6$ cells per ml for up to 6 days. The cells were plated in 96-well plates ($10^5$ cells per well) for ELISA, on glass coverslips in 24-well plates for immunocytochemistry ($5 \times 10^5$ cells per well), and in 150-$cm^2$ flasks ($2.5 \times 10^5$ cells per $cm^2$) for immunochemistry or RNA extraction. Cells were grown in the absence or presence of $10^{-11}$ to $10^{-7}$ M 1,25$(OH)_2D_3$ (provided by Milan Uskokovic, Hoffman–LaRoche).

**ELISA.** After 1–6 days of culture, cells were fixed in 3.7% formaldehyde, treated with 0.01% $H_2O_2$, and preincubated for 1 hr in phosphate-buffered saline (PBS)/3% bovine serum albumin. Cells were incubated for 2 hr with antibodies (Abs) diluted in PBS/3% bovine serum albumin with saponin (0.05%) to permeabilize the cells. After washing, cells were

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Abbreviations: 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin $D_3$; CAII, carbonic anhydrase II; Ab, antibody; mAb, monoclonal Ab; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; AMV, avian myeloblastosis virus.

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incubated with peroxidase-labeled goat anti-mouse IgG Ab (Cappel) diluted 1:1000 in PBS/3% bovine serum albumin, washed, and allowed to react with 2 mg of *o*-phenylenediamine per ml (Sigma) and 0.03% $H_2O_2$. The reaction was stopped after 15 min by the addition of $H_2SO_4$. Optical densities (ODs) were read in duplicate on an ELISA reading system (EAR400, Labinstruments) at 492/620 nm. Two monoclonal antibodies (mAbs) were used: mouse anti-chicken CAII as culture supernatant [7C6-1 (32) diluted 1:20] and anti-LEP100 as ascites fluid [(33) diluted 1:1000]. The background OD, measured without primary Ab or with an irrelevant Ab [anti-dinitrophenyl (gift of Richard Anderson, University of Texas, Dallas) diluted 1:200], was subtracted from the ODs measured with the specific Abs. In three experiments the protein contents were measured simultaneously (34) after lysis in 0.1% SDS.

**Western Blots.** Cells were washed with PBS, pelleted, and lysed at 4°C in lysis buffer [150 mM NaCl containing 1% Triton X-100, 0.2% SDS, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 10 μg of leupeptin per ml, 1 mM dithiothreitol, and 2 mM benzamidine) in 50 mM Tris buffer at pH 7.4]. Proteins were measured (34), electrophoresed through SDS/PAGE (10%), transferred to nitrocellulose (35), and incubated with a rabbit anti-chicken CAII polyclonal Ab (36) or nonimmune serum diluted 1:2000 in Tris-buffered saline. Nitrocellulose sheets were incubated for 2 hr with 0.5 μCi of $^{125}$I-labeled protein A (Amersham; 1 Ci = 37 GBq) and autoradiographed overnight (Kodak X-Omat-AT). Reactive CAII bands were quantitated with an automated densitometer (Bioimage, Ann Arbor, MI).

**Immunocytochemistry.** Cells cultured on glass coverslips were fixed for 30 min at 4°C in 3.7% formaldehyde/0.05% saponin (added to all solutions), washed, and incubated for 30 min in 0.01% $H_2O_2$. After preincubation for 20 min in 10% goat serum, the coverslips were incubated 12 hr with the mAb anti-LEP100 diluted 1:100 or anti-chicken CAII (7C6-1) culture supernatant diluted 1:5, followed by the peroxidase-labeled rabbit anti-mouse Fab fragments (Biosys, Marne la Coquette, France) diluted 1:100. Cells were counterstained with 0.1% toluidine blue in 0.1% sodium borate. Cultures were also stained for fluoride inhibitable nonspecific esterase as described (4).

**Northern Blots.** Total RNA was extracted from 6-day-old cultures by a modification of the Chirgwin method (37), separated by electrophoresis through a 1% formaldehyde/agarose gel, and transferred to Zetabind membranes (Cuno) by blotting. cDNAs were labeled with [$^{32}$P]dCTP by the random-priming method (38). Blots were probed with a 1.2-kilobase cDNA probe encoding the chicken CAII (39). Hybridization was performed by incubating the labeled cDNAs ($0.5–1.0 \times 10^6$ cpm/ml) at 42°C overnight in Thomas buffer containing 50% formamide, 50 μg/ml each of poly(A), poly(I), and poly(C) nucleotides (Boehringer Mannheim), and 8% dextran sulfate. The membranes were washed in 0.1× SSCPE (1× SSCPE = 15 mM NaCl/1.5 mM sodium citrate/1.3 mM $KH_2PO_4$/100 μM EDTA) with 0.1% SDS for 1 hr at 65°C and autoradiographed. Autoradiographic films were quantitated by a computerized densitometer scanner (Bioimage). For reprobing, the blots were washed two times at 95°C for 15 min in 0.1× SSCPE/0.1% SDS. They were successively hybridized with cDNA probes encoding chicken LEP100 (40), human α-actin (gift of Larry Kedes, Stanford, CA), and chicken genomic $\alpha^A$ globin DNA (41).

**Culture of Avian Myeloblastosis Virus (AMV)-Transformed Myelomonocytic Cell Line BM2.** BM2 cells, a line of chicken myeloblasts transformed with the AMV (clone C3A) (42), were differentiated into macrophages by stimulation with lipopolysaccharide (LPS; endotoxin of *Salmonella typhimurium*) and phorbol 12-myristate 13-acetate (PMA; Sigma) (43). BM2/C3A cells were plated at $1.0 \times 10^6$ cells per ml in medium RPMI 1640 containing 5% heat-inactivated calf serum, 5% heat-inactivated chicken serum, 50 units of penicillin per ml, and 50 μg of streptomycin per ml. The cells were cultured for 6 days in the absence or presence of 10 μg of LPS per ml and 0.25 μg of PMA per ml. $1,25(OH)_2D_3$ ($10^{-8}$ M) was added to some cultures at day 0. Cells were processed for immunocytochemistry with the mAb to CAII as described above.

## RESULTS

We analyzed the expression of CAII in bone marrow cultures by probing immunoblots with an antiserum that reacts specifically with CAII (36). A marked enhancement in the level of the immunoreactive 29-kDa CAII band was observed following 6 days of incubation in the presence of $10^{-9}$ M $1,25(OH)_2D_3$ (Fig. 1). In contrast, cells grown in the absence of $1,25(OH)_2D_3$ contained either undetectable (Fig. 1*A*) or very low (Fig. 1*B*) levels of CAII. Quantitation of the immunoreactive bands indicated that in response to $1,25(OH)_2D_3$ the levels of CAII increased from 4- to >14-fold. To determine whether this effect occurred in a dose-dependent manner, CAII levels were determined by ELISA, using a CAII-specific mAb (32), in cultures treated with various amounts of vitamin $D_3$. Expression of CAII was compared to that of LEP100, a lysosomal membrane protein (33) that was found to vary proportionally to protein content and not to levels of $1,25(OH)_2D_3$ (data not shown). As shown in Fig. 2*A*, CAII expression increased in response to increasing amounts of $1,25(OH)_2D_3$ ($10^{-11}$ to $10^{-7}$ M), both after 3 and 6 days in culture; $10^{-11}$ M $1,25(OH)_2D_3$ was sufficient to induce a significant increase in CAII levels after 6 days. A plateau was reached at $10^{-8}$ M $1,25(OH)_2D_3$ with a 3- to 4-fold increase in CAII levels after 6 days. As shown in Fig. 2*B*, the time course of CAII expression in the presence of $10^{-9}$ M $1,25(OH)_2D_3$ was also analyzed by ELISA. An increase in CAII protein levels of >2-fold was detectable 24 hr after stimulation. CAII levels increased for up to 6 days, at which point the values were ≈4 times higher than at day 1. The expression of the enzyme also increased in control cultures at day 6, probably due to the presence of low concentrations of $1,25(OH)_2D_3$ in the medium ($\approx 10^{-11}$ M). These results show that the increase in expression of CAII is induced by $1,25(OH)_2D_3$ in a dose- and time-dependent fashion and is detectable soon (≈24 hr or less) after administration of the hormone. Further, increased expression of CAII was concomitant with a marked dose-dependent decrease in [$^3$H]thymidine incorporation (data not

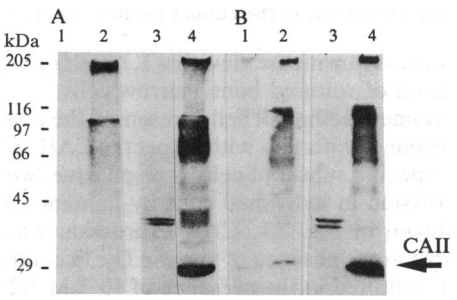


FIG. 1. Immunoblot analysis of CAII expression in bone marrow mononuclear cell lysates. Total lysates from 6-day cultures of bone marrow mononuclear cells grown in the absence (lanes 1 and 2) or in the presence (lanes 3 and 4) of $10^{-9}$ M $1,25(OH)_2D_3$ were separated on 10% SDS/PAGE and allowed to react either with nonimmune serum (lanes 1 and 3) or with the polyclonal antibody against chicken CAII (lanes 2 and 4). The 29- to 30-kDa band corresponding to CAII is undetectable in lanes 1 and 3. In lanes incubated with the immune serum, the CAII band either is undetectable (*A*, lane 2) or is present at low levels (*B*, lane 2) in the absence of vitamin $D_3$. In contrast, the CAII band is markedly enhanced (4- to >14-fold) in the presence of vitamin $D_3$ (*A* and *B*, lanes 4).

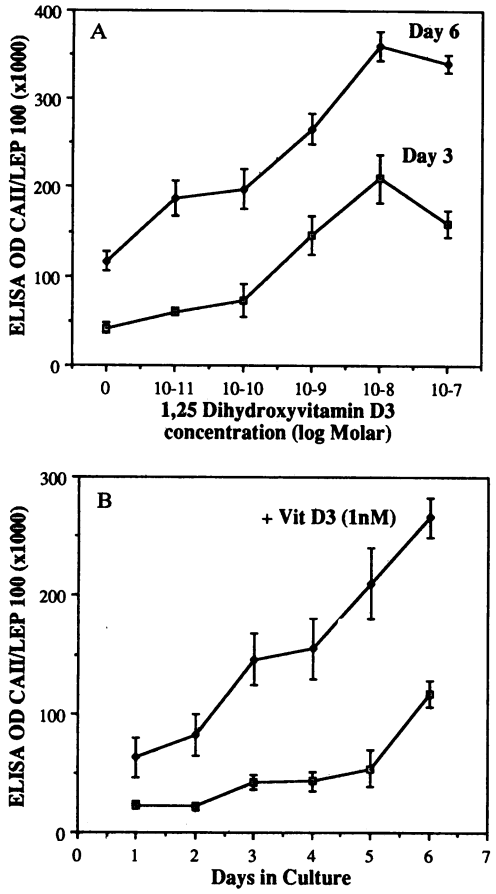


FIG. 2. ELISA analysis of CAII expression in bone marrow mononuclear cells cultured for 1–6 days in the presence of increasing concentrations of $1,25(OH)_2D_3$. ODs, measured in 4–12 experiments (each with duplicate wells), are expressed as a ratio of CAII to LEP100 expression (means ± SEM). (*A*) The level of expression of CAII, measured after 3 days (□) or 6 days (■) in culture, increased as a function of $1,25(OH)_2D_3$ concentration. The first significant effects are seen at $10^{-10}$ M $1,25(OH)_2D_3$ after 6 days and $10^{-9}$ M after 3 days. A plateau is reached at $10^{-8}$ M $1,25(OH)_2D_3$ at both time points. (*B*) At all time points, the level of expression of CAII is higher in cells cultured in the presence of $10^{-9}$ M $1,25(OH)_2D_3$ (■) than in cells cultured without $1,25(OH)_2D_3$ (□). CAII expression is already significantly increased (≈3-fold) after 24 hr of culture with vitamin $D_3$ relative to unstimulated cultures. The expression of CAII also increases in unstimulated cultures by day 6, probably due to the presence of $1,25(OH)_2D_3$ in the culture medium (about $10^{-11}$ M).

shown), consistent with the view that $1,25(OH)_2D_3$ affects the differentiation of cultured bone marrow cells.

To determine whether all cells present in the bone marrow cultures respond uniformly with respect to CAII induction or whether a specific subset of cells is responsive, we analyzed CAII expression in individual cells by immunocytochemistry. As shown in Fig. 3*A*, CAII expression was virtually undetectable in the absence of vitamin $D_3$. In contrast, many of the cells cultured in the presence of $10^{-9}$ M $1,25(OH)_2D_3$ were reactive with the CAII mAb (Fig. 3*B*). Approximately 70% of the cells exhibited detectable immunoreactivity, but with a gradient of staining intensity. The cells that were most intensely reactive appeared to represent either mononuclear cells or multinucleated cells containing low (2–10) numbers of nuclei. Most of the very large multinucleated cells (>25 nuclei) were not immunoreactive or exhibited very low levels of reactivity with the CAII mAb. A majority of the cells was also positive for nonspecific esterase, suggesting that the cultures were highly enriched for mononuclear phagocytes. We therefore investigated whether a chicken bone marrow

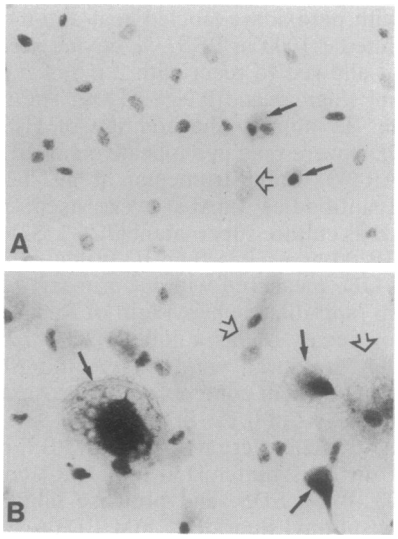


FIG. 3. Immunocytochemical localization of CAII in bone marrow mononuclear cells cultured for 6 days in the absence (*A*) or in the presence (*B*) of $1,25(OH)_2D_3$ ($10^{-9}$ M). CAII was localized with the mAb followed by peroxidase-conjugated secondary antibodies; nuclei were counterstained with toluidine blue. (*A*) In the absence of vitamin $D_3$, CAII is mostly undetectable in the cytoplasm of the cultured cells (identified by their stained nuclei); the closed arrows point to slightly positive mononuclear and binucleated cells but other binucleated cells are negative (open arrow). (*B*) In the presence of $1,25(OH)_2D_3$, the level of expression of CAII is increased in most cells, with a gradient of intensity; the closed arrows point to strongly positive mononuclear and multinucleated cells; the open arrows point to less positive multinucleated cells. (×360.)

myelomonocytic cell line could also be induced to express CAII in the presence of vitamin $D_3$. The AMV-transformed myeloblastic cell line BM2/C3A (42) was cultured in the presence of LPS and PMA to induce cell attachment and differentiation into more mature bone marrow macrophages (43). Under these conditions, the cells exhibited only moderate levels of reaction with CAII antibodies (Fig. 4*A*). However, when the cells were cultured for 6 days in the

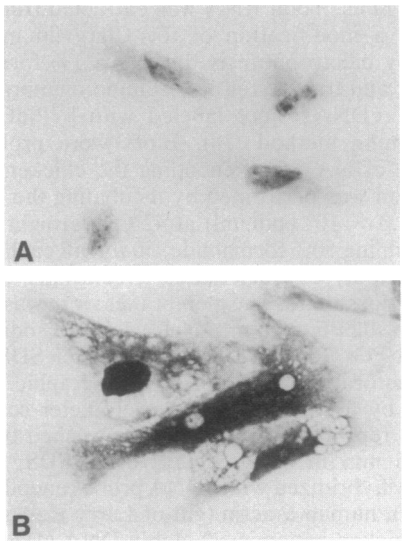


FIG. 4. Immunocytochemical localization of CAII in the AMV-transformed avian myelomonocytic cell line BM2 cultured in the presence of LPS and PMA and in the absence (*A*) or presence (*B*) of $1,25(OH)_2D_3$ ($10^{-8}$ M). (*A*) In the absence of vitamin $D_3$, CAII levels were low but still detectable in the cells induced by PMA. (*B*) In the presence of $1,25(OH)_2D_3$, the level of CAII expression was markedly increased. (×800.)

presence of $10^{-8}$ M $1,25(OH)_2D_3$, an intense staining was observed (Fig. 4*B*). More immature cells, grown in the presence of vitamin $D_3$, but in the absence of LPS and PMA induction, did not exhibit an increase in CAII expression. These results suggest that the induction of CAII expression in bone marrow cultures by $1,25(OH)_2D_3$ occurs in myelomonocytic precursors and only when cells progress through their differentiation program. $1,25(OH)_2D_3$ therefore appears to be a potent inducer of myelomonocytic cell differentiation, possibly committing these cells toward the osteoclast lineage.

Northern blot analysis was used to analyze CAII mRNA expression in vitamin $D_3$-treated cells. Mononuclear cells isolated from bone marrow were exposed to concentrations of $10^{-11}$ to $10^{-8}$ M $1,25(OH)_2D_3$ for 6 days. As shown in Fig. 5, $1,25(OH)_2D_3$ stimulated CAII mRNA levels in a dose-dependent fashion. In contrast, unstimulated cultures contained only low levels of CAII mRNA. To ensure that equal amounts of RNA were loaded, the blots were sequentially probed with cDNA encoding LEP100 and $\alpha$-actin. Actin mRNA decreased in response to $1,25(OH)_2D_3$, whereas LEP100 mRNA levels remained constant (Fig. 5). By normalizing to LEP100 mRNA, we calculated that CAII mRNA levels increased from 1.6 times at $10^{-11}$ M $1,25(OH)_2D_3$ to 7.2 times at $10^{-8}$ M. These results show that treatment of bone marrow mononuclear cells with $1,25(OH)_2D_3$ results in a specific increase in CAII mRNA levels. The regulation of CAII expression by $1,25(OH)_2D_3$ therefore occurs at pretranslational levels.

Northern blots were also probed with cDNA encoding $\alpha^A$-globin. No hybridization could be detected in the bone marrow cultures when control erythroid cells were positive (data not shown). This result suggests that $1,25(OH)_2D_3$ induction of CAII is specific for the nonerythroid cell population and does not occur in cells of the erythroid lineage. To determine whether the levels of CAII expression in these cultures could be affected by other members of the steroid family of hormones, we examined the effects of retinoic acid, a steroid-like factor known to interact with steroid hormone-responsive elements. Retinoic acid ($10^{-7}$ to $10^{-9}$ M) had no detectable stimulating effects on the levels of CAII mRNA or protein expression (data not shown). This result suggests that $1,25(OH)_2D_3$ is a specific inducer of CAII expression in nonerythroid bone marrow cells.

## DISCUSSION

CAII is an enzyme that is involved in acid–base regulation. It is expressed at high levels in the gastric oxyntic cell (27), the kidney intercalated cell (26), and the osteoclast (21, 22), cell types that are all involved in acid secretion (26, 27, 44). Our results indicate that $1,25(OH)_2D_3$ induces the expression of CAII in myelomonocytic cells. Although the osteoclast is a member of this cell lineage (2, 4–6), other myelomonocytic cells, including monocytes and macrophages, express only low levels of CAII (24). The specific induction of CAII expression in bone marrow nonerythroid cells suggests that $1,25(OH)_2D_3$ plays an important role in the differentiation of myelomonocytic precursors and their commitment toward the osteoclast lineage. A number of studies have shown that $1,25(OH)_2D_3$ stimulates the formation of osteoclast-like cells in long-term bone marrow cultures (13, 19, 20) and can induce the complete differentiation of some of these cells into functional osteoclasts (14). Further, we have shown that late mononuclear precursors of the osteoclast express high levels of CAII (45) and that expression of CAII in short-term bone marrow cultures is concomitant with that of other markers of the mature osteoclast (46), such as the $Na^+,K^+$-ATPase (47) and the osteoclast functional antigen, a vitronectin receptor (48). Interestingly, we detect significant changes in the expression of these markers of osteoclast differentiation within 24 hr of exposure to $1,25(OH)_2D_3$. In contrast, phenotypic and functional changes characteristic of osteoclast differentiation have been reported to require weeks in culture (13, 14). Taken together, our results suggest that $1,25(OH)_2D_3$ regulates the expression of carbonic anhydrase as well as that of other osteoclast markers (46), inducing myelomonocytic cells in the bone marrow to differentiate toward osteoclasts. As reported for newborn monocytes (20), we did not, however, observe actual bone resorption (in the form of resorption lacunae) when bone marrow mononuclear cells were cultured on devitalized bone slices, even after 2 weeks in the presence of $1,25(OH)_2D_3$ (unpublished observations). In contrast, formation of bone resorbing osteoclasts was obtained with cultures of total bone marrow (14), including stromal cells, which are an important source of colony-stimulating factors (49). $1,25(OH)_2D_3$ alone is therefore most likely not sufficient to induce the formation of functional cells, even in the presence of bone matrix.

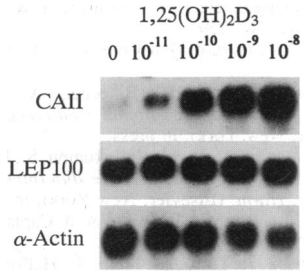


FIG. 5. Northern blot analysis of CAII mRNA expression in bone marrow mononuclear cells. Bone marrow mononuclear cells were cultured for 6 days in the absence or in the presence of increasing concentrations of $1,25(OH)_2D_3$. Total mRNA was extracted, electrophoresed on agarose gels, blotted to Zetabind membranes, and hybridized with radiolabeled cDNA probes encoding the chicken CAII; after stripping, the membranes were reprobed with radiolabeled cDNA probes encoding LEP100 and $\alpha$-actin. A marked dose-dependent increase in CAII mRNA expression was observed in the presence of $1,25(OH)_2D_3$ [from 1.6 times LEP100 values at $10^{-11}$ M to 7.2 times at $10^{-8}$ M $1,25(OH)_2D_3$]. In contrast, LEP100 mRNA levels remained stable and actin mRNA levels decreased.

Important issues in osteoclast differentiation are whether these cells share a common precursor with monocytes and macrophages and at what stage(s) of differentiation mononuclear phagocytes can be committed to the osteoclast phenotype. Although the cultures we have used are purified by sedimentation and adherence, they are relatively heterogeneous for cell types. It was therefore not possible to directly determine if a particular cell type specifically expressed CAII in response to $1,25(OH)_2D_3$. Many cells reacted positively with monoclonal antibodies to CAII after stimulation with $1,25(OH)_2D_3$, albeit with a gradient of intensity. A number of lines of evidence, however, suggest that the responsive cells are likely to be committed to the mononuclear phagocytic lineage. First, the isolation procedures and culture conditions that we have used are known to markedly enrich for cells in this lineage (5, 13, 31, 35). Second, $1,25(OH)_2D_3$ also induced the expression of CAII in a homogeneous marrow-derived avian myelomonocytic cell line (BM2), a result consistent with the recent report of Shapiro *et al.* (30) in the transformed human promonocytic cell line HL60. Therefore, both transformed and nontransformed bone marrow myelomonocytic cells respond to vitamin $D_3$ with an increase in CAII expression.

Regarding the second issue, a number of independent observations would suggest that it is only at a stage intermediate between the most immature myelomonocytic precursors and the more mature circulating monocytes that cells can respond to $1,25(OH)_2D_3$ and, possibly, further differentiate into osteoclasts. First, we report here that BM2 cells can express CAII at high levels only in the presence of both LPS

and PMA and $1,25(OH)_2D_3$. In the absence of $1,25(OH)_2D_3$, BM2 cells differentiate into macrophages (43) but do not express high levels of CAII. Reciprocally, treatment of cells with $1,25(OH)_2D_3$ alone ($10^{-8}$ M) did not induce the expression of detectable levels of CAII. This would suggest that these myelomonocytic cells must pass through an LPS/PMA sensitive step in order to acquire CAII responsiveness to vitamin $D_3$. Second, mature macrophages and peripheral blood monocytes do not express this enzyme, whereas more immature monocytes isolated from newborn umbilical chord do (24). Third, only early stages of differentiation in the monocytic series form multinucleated cells (19, 50) and express the osteoclast vitronectin receptor in response to $1,25(OH)_2D_3$ (20).

The induction of CAII mRNA expression by $1,25(OH)_2D_3$ suggests that this steroid hormone may regulate transcription of the CAII gene. We therefore analyzed the promoter region of the CAII gene (39) for putative consensus sequences for vitamin D-responsive elements (VDRE). This analysis did not indicate the presence of a region identical to the VDRE described for the rat or the human osteocalcin genes (51, 52). However, there are imperfect palindromic sequences in the promoter region of the chicken CAII gene [GGTGA (G) TGAAC and GGTGA (C) TCAAC], which are very similar to the reported VDRE and are compatible with steroid hormone-responsive elements (53) and located in positions compatible with the activation of gene transcription (700–750 base pairs upstream from the TATA boxes). It will clearly be of interest to determine whether the complex formed by $1,25(OH)_2D_3$ with its receptor binds to and directly activates the chicken CAII gene.

In conclusion, our results suggest that vitamin $D_3$ acts directly on myelomonocytic cells to induce the expression of carbonic anhydrase. This gene is expressed at high levels in the fully differentiated osteoclast and its late precursors and is necessary for its function in bone resorption (28).

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