

# Carbonic anhydrase II is induced in HL-60 cells by 1,25-dihydroxyvitamin D<sub>3</sub>: a model for osteoclast gene regulation

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Carbonic anhydrase II (CA II) generates the H<sup>+</sup> required for osteoclast-mediated bone resorption in humans. We have developed the human promyelocytic cell line HL-60 as a model system with which to study the osteoclast-specific expression of the CA II gene. Treatment of the cell line with 1,25-dihydroxyvitamin D<sub>3</sub> resulted in a dramatic *de novo* induction of CA II at both the protein and mRNA levels. CA II mRNA was also induced to a lesser extent by 12-*O*-tetradecanoyl phorbol 13-acetate. Treatment with dimethyl sulfoxide did not increase CA II mRNA. These findings indicate that the HL-60 cell line will be a useful model system to study the osteoclast-specific expression of the CA II gene.

Carbonic anhydrase II; Osteoclast; Dihydroxyvitamin D<sub>3</sub>, 1,25-; (HL-60 cell)

## 1. INTRODUCTION

Carbonic anhydrase (CA, EC 4.2.1.1) catalyzes the interconversion of carbon dioxide and water to bicarbonate and protons. A considerable amount of information is known about the molecular structures, genetics, evolution, and physiological roles of the different CA isozymes [1]. In mammals, the CA isozymes are coded by at least seven genes, designated CA I–CA VII, which in general have different tissue specificities and probably varying physiological roles [2]. In humans, an inherited deficiency of CA II (CA II deficiency syndrome) is characterized by osteopetrosis, renal tubular acidosis, and cerebral calcification [3] and in mice by renal tubular acidosis [4] and vascular calcifications [5]. The osteopetrosis in CA II deficiency syndrome is an osteoclast-specific defect as

shown by two lines of evidence. CA II in bone is found only in osteoclasts in a variety of species [6–10], and variation in the activity of CA II caused by hormonal treatment (e.g. parathyroid hormone) in bone culture influences the degree of bone resorption [11].

Because the study of CA II gene regulation at the molecular level is hampered by the difficulty of obtaining a sufficient number of purified osteoclasts, a model cell culture system is needed. Since there is a close developmental relationship between osteoclasts and macrophages and monocytes [12], it might be possible to study CA II gene regulation in a cell line derived from the latter cell types. The HL-60 cell line, derived from a patient with promyelocytic leukemia, can be induced to undergo monocytic differentiation by a variety of compounds [13] including 1,25-dihydroxyvitamin D<sub>3</sub> [1,25-(OH)<sub>2</sub>D<sub>3</sub>], a hormone known to affect strongly bone resorption [14]. Trace levels of CA II have been found in uninduced HL-60 cells [15]. We reasoned that this cell line might provide a potential model for the regulation of the CA II gene in the osteoclast, and that CA II might be induced to higher levels by exposing the cells to 1,25-(OH)<sub>2</sub>D<sub>3</sub>. It is shown here that

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such an induction does occur for both CA II mRNA and protein.

## 2. MATERIALS AND METHODS

### 2.1. Cell culture and treatments

HL-60 cells were grown in RPMI-1640 medium with 10% fetal calf serum. Cells were induced with 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), or dimethyl sulfoxide (DMSO) for varying lengths of time as described in the figure legends. The concentrations were 10 nM 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 10 nM TPA and 1.25% DMSO.

### 2.2. Northern blots

RNA was isolated by a guanidine-HCl procedure [16]. RNAs (10 µg per lane, except as noted) were run in formaldehyde gels and transferred to nylon membranes by capillary blotting [17]. The amount of RNA loaded was confirmed by ethidium bromide staining. Blots were probed with randomly primed [18] human CA II cDNA [19], washed and autoradiographed. Some blots were also probed with human CA I [20], CA III [21], and CA VII DNAs [22].

### 2.3. Western blots

Cell lysates were prepared by freeze-thawing cells in phosphate-buffered saline and pelleting the debris. 50 µg protein per lane were run on SDS gels, transferred to a nylon membrane [23], and probed with rabbit anti-human CA II followed by goat anti-rabbit IgG conjugated to alkaline phosphatase. Blots were developed with nitroblue tetrazolium and bromochloroindolyl phosphate [24].

### 2.4. Cellulose acetate electrophoresis

Human CA I and CA II were purified from hemolysates by affinity chromatography as described [25]. Cellulose acetate electrophoresis, using a buffer composed of 0.44 M Tris, 50 mM boric acid, and 15 mM EDTA at pH 9.1, was performed at 300 V for 3 h [26]. Carbonic anhydrase activity was detected by a CO<sub>2</sub>-driven indicator (bromothymol blue) color change as described in [27].

## 3. RESULTS

### 3.1. CA II mRNA is induced by 1,25-dihydroxyvitamin D<sub>3</sub>

The treatment of HL-60 cells with 1,25-(OH)<sub>2</sub>D<sub>3</sub> or TPA, another reagent commonly used to induce HL-60 cells along a monocytic differentiation pathway, results in the induction of CA II mRNA after 48 h incubation (fig.1). However, treatment of the cells with DMSO, which causes HL-60 cells to undergo granulocytic differentiation [13], does not result in induction of CA II mRNA. Densitometric scans of the lanes give estimates of a 5-fold increase of TPA-induced CA II message and a 20-fold increase with 1,25-(OH)<sub>2</sub>D<sub>3</sub> after 48 h.

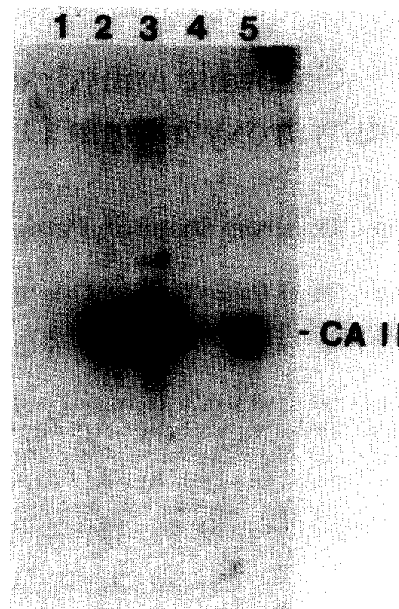


Fig.1. Northern blot showing effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub>, TPA, and DMSO on CA II induction in HL-60 cells. All lanes contained 10 µg total RNA except lane 3 which had 25 µg total RNA. HL-60 cells were treated for 48 h with each inducing agent at the concentrations given in section 2. Lanes: (1) uninduced, (2,3) induced with 1,25-(OH)<sub>2</sub>D<sub>3</sub>, (4) induced with DMSO, (5) induced with TPA. The blot was probed with the cloned human CA II cDNA [14].

The blot was also probed with human CA I, CA III, and CA VII DNAs. No signal was obtained with any of these probes (not shown). Probes for other CA isozymes are not yet available.

### 3.2. Rate of CA II mRNA induction

The time course of the change in CA II mRNA levels in HL-60 cells was determined after treatment with TPA, 1,25-(OH)<sub>2</sub>D<sub>3</sub>, and DMSO. The CA II mRNA levels in TPA-treated cells appear to peak at 48 h and decrease slightly at 72 and 96 h (fig.2). No increase in CA II mRNA was detected after 3 h exposure to 1,25-(OH)<sub>2</sub>D<sub>3</sub>; however, an increase was detected at 24 h, which continued to rise up to 96 h (fig.2). CA II mRNA levels were not observed to change from control levels throughout 96 h of treatment with DMSO (not shown).

### 3.3. CA II protein is induced by 1,25-dihydroxyvitamin D<sub>3</sub>

Several Western blots of treated HL-60 cell lysates were carried out, and a representative blot

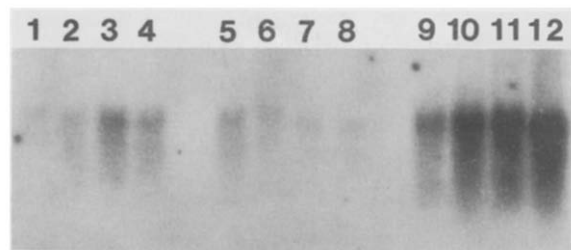


Fig.2. TPA and 1,25-(OH)<sub>2</sub>D<sub>3</sub> time courses of CA II mRNA induction in HL-60 cells shown by Northern blot analysis. Cells were induced under standard conditions as described in the text and harvested at various times. Lanes: (1,6) control cells, (2-5) treated with TPA, (7-12) treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub>. Times of treatment: (2,9) 24 h, (3,10) 48 h, (4,11) 72 h, (5,12) 96 h, (7) 1 h, (8) 3 h.

is shown in fig.3. A trace amount of CA II protein can be seen in control cells, and an equivalent amount can be observed in 72 and 96 h DMSO-treated lysates. In contrast, an approx. 10-fold increase in CA II protein can be seen in 72 and 96 h 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced lysates. No increase in CA II protein could be detected at 48 h of treatment with 1,25-(OH)<sub>2</sub>D<sub>3</sub> (not shown). When compared to known amounts of purified human CA II, it was estimated that 50 ng CA II was present at 96 h of induction in 50 µg soluble protein.

### 3.4. CA II appears to be the only CA in HL-60 cells

To confirm the identity of the isozyme as CA II, lysates were run on a cellulose acetate membrane and stained for carbonic anhydrase activity (fig.4). An increase in activity can be detected at 72 and 96 h in a band that co-migrates with CA II, but not with CA I. No increase in CA II activity can be

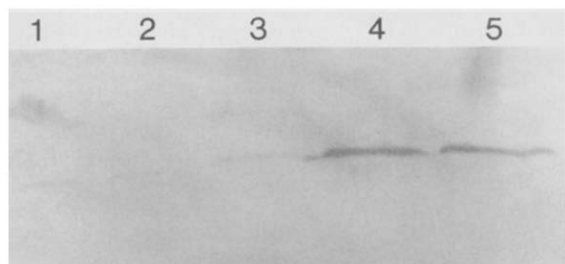


Fig.3. Western blot of cell lysates from DMSO- and 1,25-(OH)<sub>2</sub>D<sub>3</sub>-treated cells. 50 µg protein were loaded per lane. (1) Control cells; (2) DMSO, 72 h; (3) DMSO, 96 h; (4) 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 72 h; (5) 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 96 h.

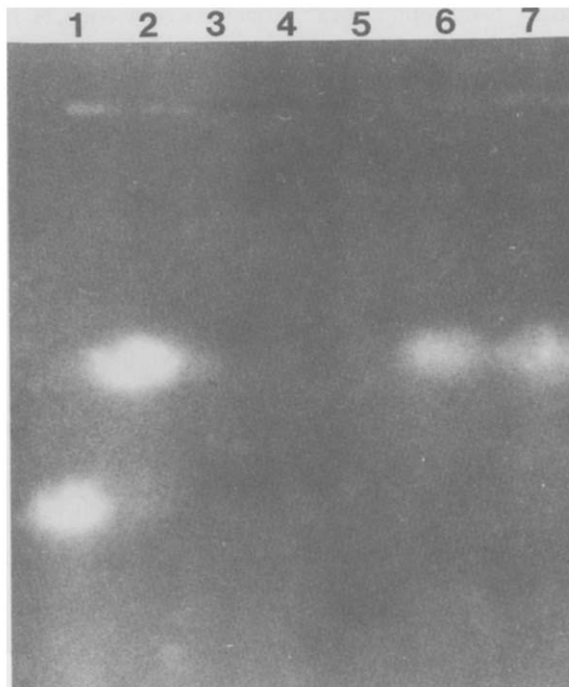


Fig.4. Cellulose acetate electrophoresis of cell lysates stained for carbonic anhydrase activity at various times of treatment with 1,25-(OH)<sub>2</sub>D<sub>3</sub>. Lanes: (1) purified human CA I control; (2) purified human CA II control. Remaining lanes of induced lysate: (3) 0 h, (4) 24 h, (5) 48 h, (6) 72 h, (7) 96 h.

observed at 48 h (not shown). CA III migrates to another position in this system, but because of its substantially lower carbonic anhydrase activity [28], it was not included in this experiment.

## 4. DISCUSSION

We have developed a model cell culture system which can be used to study the osteoclast-specific regulation of the human CA II gene. HL-60 cells respond to a variety of hormones that also effect bone metabolism [12,13]. The effect of these hormones on the regulation of the CA II gene can now be studied at the molecular level. Although HL-60 cells express CA II, this is not so in cultured rat peripheral monocytes, and most types of macrophages [8]. Considering the close developmental relationships between osteoclasts and monocytes and macrophages, as well as the sometimes aberrant nature of genetic programs of neoplastic cells, these results are not surprising. Both osteoclasts

and HL-60 cells appear to express only the CA II isozyme.

The human and mouse CA II genes have been cloned and their promoter regions sequenced [29]. The promoter lies within a GC-rich island, and the minimal elements required for high-level expression in human HeLa cells and mouse L cells have been identified [30]. However, preliminary experiments in which the minimal promoter has been transfected into HL-60 cells has shown that, although the TPA-responsive element is present, the 1,25-(OH)<sub>2</sub>D<sub>3</sub>-responsive element lies outside of this region (L.H.S. and P.J.V., unpublished).

Since little is known about how 1,25-(OH)<sub>2</sub>D<sub>3</sub> affects the expression of genes at the molecular level, this model system should provide some valuable insights into the regulatory effects of this hormone. Once the regulation of the CA II gene is understood in HL-60 cells, critical experiments can be designed to examine its regulation in limited amounts of purified osteoclasts. In addition, the results obtained may be useful for developing a successful gene replacement therapy of the recently described CA II-deficient mouse model [4].

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