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Regulation of metallothionein gene expression by 1α,25-dihydroxyvitamin D_3 in cultured cells and in mice

(vitamin D_3/epidermal keratinocytes)

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ABSTRACT 1α,25-Dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], a hormonally active form of vitamin D_3, has been shown to modulate cell differentiation and tumor promotion. This report demonstrates that mRNA of the metallothionein (MT) gene was induced by 1α,25$(OH)_2D_3$ in cultured epidermal keratinocytes and also in liver, kidney, and skin tissues when 1α-hydroxyvitamin D_3, a synthetic precursor of 1α,25$(OH)_2D_3$, was applied *in vivo*. Exposure of FRSK cells, a cell line derived from fetal rat skin keratinocytes, to 1α,25$(OH)_2D_3$ at 5 ng/ml (12 nM) increased MT mRNA to almost the same extent as that induced by 10 μM dexamethasone or 1 μM $CdCl_2$. This increase in the level of MT mRNA was evident within 2 hr and was maximal 12–24 hr after the addition of 1α,25$(OH)_2D_3$. The induction was dose-dependent with concentrations of 1α,25-$(OH)_2D_3$ from 0.05 to 5.0 ng/ml. Amounts of MT increased with the increase of MT mRNA induced by 1α,25$(OH)_2D_3$. Of the derivatives of vitamin D_3 tested, only 1α,25$(OH)_2D_3$ caused marked induction. Treatment with cycloheximide did not inhibit MT mRNA induction by 1α,25$(OH)_2D_3$. 1α,25$(OH)_2D_3$ induced MT mRNA in primary cultures of mouse epidermal keratinocytes but not in IAR-20, a liver cell line. 1α,25$(OH)_2D_3$ had a similar effect *in vivo*: oral administration of 1α-hydroxyvitamin D_3 to mice resulted in increased levels of MT mRNA in the liver, kidney, and skin 24 hr later. Increase in the level of MT mRNA may be relevant to some biological actions of 1α,25$(OH)_2D_3$.

Metallothioneins (MTs) are cysteine-rich, low molecular weight proteins that selectively bind heavy metal ions, such as cadmium and zinc. Although they are ubiquitously distributed, physiological functions of MTs are not fully understood (1). The induction of MTs by heavy metals and the binding of MT to heavy metals suggest MTs protect against heavy-metal toxicity. Acute-phase responses elicited by lipopolysaccharide, interleukin 1, or interferon, for example, lead to increased synthesis of MTs, suggesting a protective effect against stressful stimuli. Furthermore, MTs are reported to be scavengers of free radicals (2). Another function suggested for MTs is the regulation of trace metal metabolism, especially maintenance of zinc homeostasis (3).

In our laboratories (T.S. and T.K.), we have been working on the control of cellular differentiation and tumor promotion by 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], a hormonally active form of vitamin D_3 regulating the plasma calcium level (for review, see ref. 4). We have reported that 1α,25$(OH)_2D_3$ modulates differentiation of alveolar macrophages (5), leukemic cells (6, 7), epidermal keratinocytes (8), and melanoma cells (9). Furthermore, we found that 1α,25$(OH)_2D_3$ inhibited tumor promotion by phorbol 12-*O*-tetradecanoate 13-acetate (TPA) and mezerein in mouse skin (10). It also inhibited induction of ornithine decarboxylase by tumor promoters in the skin, stomach, colon, and liver (11, 12). In cell culture systems, however, this hormone was found to enhance chemical transformation of BALB 3T3 (13, 14) and Syrian hamster cells (15) and also to induce anchorage-independent growth of JB6 (16), BALB 3T3, and NIH 3T3 (17) cells. The apparent discrepancy between its effects on tumor promotion *in vitro* and *in vivo* requires further study, especially at the molecular level.

Identification of genes that are regulated by 1α,25$(OH)_2D_3$ and the mechanisms of its regulatory effect are of particular importance. Calcium binding protein was shown to be induced in rat intestine by 1α,25$(OH)_2D_3$ (18). DeLuca and his colleagues (19) reported that four distinct mRNAs were significantly increased in rat intestine when 1α,25$(OH)_2D_3$ was applied to vitamin D-deficient animals: these were vitamin D-dependent calcium-binding protein, mitochondrial ATP synthetase, and cytochrome oxidase subunits I and III. We found (17) that 1α,25$(OH)_2D_3$ induced expression of the c-Ki-*ras* gene in BALB 3T3 and NIH 3T3 cells.

In this study, we found that mRNA of the MT gene was induced by 1α,25$(OH)_2D_3$ in cultured epidermal keratinocytes and also in liver, kidney, and skin tissues when 1α-hydroxyvitamin D_3 [1α$(OH)D_3$], a synthetic precursor of 1α,25$(OH)_2D_3$, was applied *in vivo*. This induction of MTs may be relevant to some biological actions of 1α,25$(OH)_2D_3$.

MATERIALS AND METHODS

Chemicals. Vitamin D_3 and its derivatives, 1α,25$(OH)_2D_3$, 1α$(OH)D_3$, (24*R*)-25-dihydroxyvitamin D_3, and 25-hydroxyvitamin D_3, were supplied by Chugai Pharmaceutical (Tokyo). Dexamethasone was purchased from Sigma. [23,24-^{3}H]1α,25$(OH)_2D_3$ (specific activity, 83 Ci/mmol; 1 Ci = 37 GBq) and [^{3}H]leucine (131 Ci/mmol) were obtained from Amersham. TPA was purchased from Consolidated Midland (Brewster, NY). Vitamins were dissolved in ethanol for addition to cultured cells. For administration to animals, 1α$(OH)D_3$ was dissolved in medium-chain triacylglycerol (Nisshin Seiyu, Tokyo). TPA was used as a solution in acetone. All other compounds including heavy-metal salts were of the purest grade available.

Cells and Cell Culture. The cells used were FRSK [a cell line derived from epidermal keratinocytes of fetal rats (20)], IAR-20 [a cell line derived from rat liver parenchymal cells (21)], and primary cultures of epidermal keratinocytes of newborn Sencar mice [a strain of mice that is highly sensitive

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Abbreviations: MT, metallothionein; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; TPA, phorbol 12-*O*-tetradecanoate 13-acetate; 1α$(OH)D_3$, 1α-hydroxyvitamin D_3.

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to two-stage carcinogenesis (22)]. These cells were grown in Eagle's minimal essential medium supplemented with 10% (vol/vol) fetal calf serum at 37°C in an atmosphere of 5% CO_2/95% air. Isolation of mouse epidermal cells was described elsewhere (23).

Animal Experiments. Female 7- to 9-week-old Sencar mice, raised in our laboratory, were used. $1\alpha(OH)D_3$ was given by gastric tube at 5 μg per animal as a solution in 50 μl of medium-chain triacylglycerol. Control mice were treated with the solvent alone. After appropriate times, mice were killed by cervical dislocation; and their liver, kidney, and dorsal skin were removed. Liver and kidney were washed with ice-cold isotonic phosphate-buffered saline (PBS) by perfusion to remove blood. The area of skin removed had been shaved and depilated, and, after excision of the skin sheet, its inside surface was scraped with a glass slide to remove fat and subcutaneous tissues. Tissues were minced and homogenized in a Polytron mixer in 4 M guanidine thiocyanate mixture (24).

Extraction and Analysis of RNA. Total cellular RNA was extracted from homogenized cells or tissues by the guanidine thiocyanate/hot-phenol method and then separated on 1.5% agarose gel containing 2.2 M formaldehyde (24). Usually, 100–200 μg of total RNA were obtained from 10^7 cells, and 10–50 μg of it was loaded on gel. RNA was transferred to a nitrocellulose filter and hybridized with ^{32}P-labeled probes, followed by autoradiography. The probe for the MT gene was the 0.4-kilobase-pair *Eco*RI–*Hin*dIII fragment of $m_1pEH.4$ derived from mouse MT-I (25). The amount of RNA was monitored by the use of a 1.4-kilobase-pair *Bam*HI fragment of the β-actin cDNA clone (26).

Assay of Receptors for $1\alpha,25(OH)_2D_3$. Receptors for $1\alpha,25(OH)_2D_3$ were assayed by incubation of the cytosol fraction with $[23,24\text{-}^3H]1\alpha,25(OH)_2D_3$ in the presence and absence of a large excess of unlabeled $1\alpha,25(OH)_2D_3$ as described elsewhere (8).

RIA of MT. The cells (5×10^6 cells) were homogenized in 125 mM borate·NaOH, pH 8.2/75 mM NaCl using a Teflon homogenizer and centrifuged at 10,000 × *g* for 5 min. The supernatant was heated at 70°C for 3 min, centrifuged, and subjected to RIA with sheep antiserum against rat hepatic MT-I and ^{125}I-labeled rat hepatic MT-II. Details of RIA were reported elsewhere (27).

RESULTS

Induction of MT mRNA by $1\alpha,25(OH)_2D_3$ in FRSK Cells. Cultures of FRSK cells consisted of polygonal epithelial cells with the typical appearance of keratinocytes and showed focal stratification when cultured for a longer period (20). We found that FRSK cells contained a specific receptor for $1\alpha,25(OH)_2D_3$ with a K_d value of 39 pM and N_{max} of 63 fmol/mg of protein (where N_{max} is maximal binding capacity). The presence of phorbol ester binding sites and their characteristics in this cell line were reported elsewhere (28).

During a survey of cell lines for the study on possible involvement of MTs in x-ray resistance, we found incidentally that in FRSK cells significant MT mRNA was induced in response to $1\alpha,25(OH)_2D_3$ (Fig. 1). Exposure of FRSK cells to $1\alpha,25(OH)_2D_3$ at 5 ng/ml (12 nM) for 7 hr increased the MT mRNA level from 5- to 10-fold of the control level, which is almost the same as seen with 10 μM dexamethasone or 1 μM $CdCl_2$, known inducers of MTs. No induction of MT mRNA was observed when the cells were treated with 5 μM $MnCl_2$, with 5 μM $CuCl_2$, or with TPA at 100 ng/ml.

In FRSK cells, MT mRNA was induced by $1\alpha,25(OH)_2D_3$ in a time- and dose-dependent manner. The level was increased within 2 hr and reached a maximum at 12–24 hr (Fig. 2). As shown in Fig. 3, induction of MT mRNA was dose-dependent with concentrations of $1\alpha,25(OH)_2D_3$ from 0.05 to 5.0 ng/ml, the usual dose range for its biological effects in cultured cells.

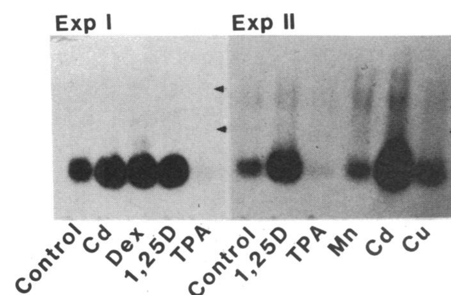

FIG. 1. Induction of MT mRNA by $1\alpha,25(OH)_2D_3$ and other inducers in FRSK epidermal cell line. Subconfluent cultures were treated for 7 hr with $1\alpha,25(OH)_2D_3$ (1,25D) at 5 ng/ml (12 nM), TPA at 100 ng/ml (162 nM), 10 μM dexamethasone (Dex), 1 μM $CdCl_2$ (Cd), 5 μM $MnCl_2$ (Mn), or 5 μM $CuCl_2$ (Cu). Total cellular RNA was isolated and examined by the conventional RNA gel blotting method with a 0.4-kilobase-pair *Eco*RI–*Hin*dIII fragment of $m_1pEH.4$ derived from mouse MT-I as probe. Upper and lower arrowheads indicate the positions of 28S and 18S rRNA, respectively. The level of β-actin mRNA was not affected by treatments with these inducers in this or subsequent experiments (data not shown). Exp, experiment.

The major transcript of the MT gene was about 0.5 kilobase (kb) long, but a weak band of a larger transcript of 2–4 kb, which was probably a precursor of MT mRNA, was also seen. Subgroups of MTs could not be resolved, because the probe we used covered almost the whole sequence of MT cDNA.

For assessing the specificity of the effect of $1\alpha,25(OH)_2D_3$, FRSK cells were treated for 7 hr with other derivatives of vitamin D_3—i.e., (24*R*)-25-dihydroxyvitamin D_3 [a metabolite formed in the kidney in parallel with $1\alpha,25(OH)_2D_3$], 25-hydroxyvitamin D_3 [a precursor of $1\alpha,25(OH)_2D_3$ formed in the liver], $1\alpha(OH)D_3$, and vitamin D_3. All these derivatives induced MT mRNA, but their effects were much less and were only observed at higher concentrations (50 or 500 ng/ml) (data not shown), indicating the specific effect of $1\alpha,25(OH)_2D_3$.

To exclude the possibility that $1\alpha,25(OH)_2D_3$ induced MT mRNA as a secondary response mediated by expression of another gene, we treated FRSK cells for 7 hr with $1\alpha,25(OH)_2D_3$ at 5 ng/ml in the presence of cycloheximide at 5 μg/ml, which caused 92% inhibition of protein synthesis as measured by incorporation of $[^3H]$leucine. We found that cycloheximide did not interfere with $1\alpha,25(OH)_2D_3$-induced MT mRNA accumulation, indicating that $1\alpha,25(OH)_2D_3$ regulates expression of the MT gene directly.

Induction of MT mRNA by $1\alpha,25(OH)_2D_3$ in Mouse Epidermal Cells. We examined the response of the MT gene to $1\alpha,25(OH)_2D_3$ in primary cultures of mouse epidermal keratinocytes and cultures of IAR-20 cells (Fig. 4). MT mRNA was induced in response to $1\alpha,25(OH)_2D_3$ in primary cultures

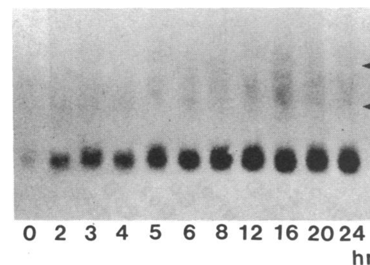

FIG. 2. Time dependence of induction of MT mRNA by $1\alpha,25(OH)_2D_3$ at 5 ng/ml in FRSK cells. Conditions were as for Fig. 1. Lanes, time (hr) of induction.

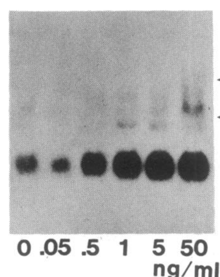

FIG. 3. Dose dependence of induction at 7 hr of MT mRNA in FRSK cells by 1α,25(OH)$_2$D$_3$. Conditions were as for Fig. 1. Lanes, concentration (ng/ml) of 1α,25(OH)$_2$D$_3$.

of mouse epidermal keratinocytes but to a lesser extent than with CdCl$_2$. In IAR-20 cells, induction of MT mRNA was observed with TPA and CdCl$_2$ but not with 1α,25(OH)$_2$D$_3$. Because IAR-20 cells contained a specific receptor for 1α,25(OH)$_2$D$_3$ with a K_d value of 11.4 pM and N_{max} of 24.4 fmol/mg of protein, the absence of the induction in this cell line was not attributable to the absence of its receptor.

Increased Synthesis of MT in 1α,25(OH)$_2$D$_3$-Treated Cells. Amounts of MT were quantitated by RIA using sheep antiserum against rat hepatic MT-I (27). When FRSK cells were treated with 1α,25(OH)$_2$D$_3$ at 5 ng/ml, the amount of MT was increased with time of exposure (Fig. 5): the cells treated for 24 hr contained $\approx$260 pg of MT per mg of protein, which is $\approx$4-fold that of untreated cells. The same extent of increased synthesis of MT was also obtained on treatment with 10 μM dexamethasone for 12 hr (Fig. 5). These results indicate that 1α,25(OH)$_2$D$_3$ induces MT mRNA that in turn is used to increase synthesis of MT.

Induction of MT mRNA *in Vivo* by Oral Administration of 1α(OH)D$_3$. Next, we examined whether 1α,25(OH)$_2$D$_3$ also induced MT mRNA *in vivo*. For this purpose, we administered 1α(OH)D$_3$ to mice at 5 μg per animal by gastric tube. 1α(OH)D$_3$ is known to be converted to 1α,25(OH)$_2$D$_3$ in the liver without hormonal control and so should evoke the systemic effects, if any, of 1α,25(OH)$_2$D$_3$ (29, 30). Indeed, we have found (12) that 12 hr after intragastric administration of 1α(OH)D$_3$ the plasma level of 1α,25(OH)$_2$D$_3$ increased to a peak of $\approx$18 times the control level and that this was followed by hypercalcemia of $\approx$14 mg of calcium per dl on day 2–3.

We measured the MT mRNA levels in three tissues: liver and kidney, because these are major tissues for synthesis of MTs, and skin, because we had demonstrated induction of MT mRNA in cultured skin keratinocytes. As shown in Fig. 6, MT mRNA was induced in all these tissues by treatment of the mice with 1α(OH)D$_3$. The induction was evident 24 hr after administration of 1α(OH)D$_3$ and reached a maximum level at 48–72 hr. No induction was observed 2, 6, or 12 hr after 1α(OH)D$_3$ administration (data for 2 and 6 hr not

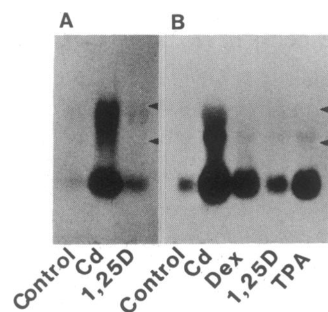

FIG. 4. Induction of MT mRNA by 1α,25(OH)$_2$D$_3$ and other inducers in primary culture of mouse epidermal keratinocytes (*A*) and cultures of IAR-20 rat liver cells (*B*). The cells were treated as described in Fig. 1. Abbreviations are as in Fig. 1.

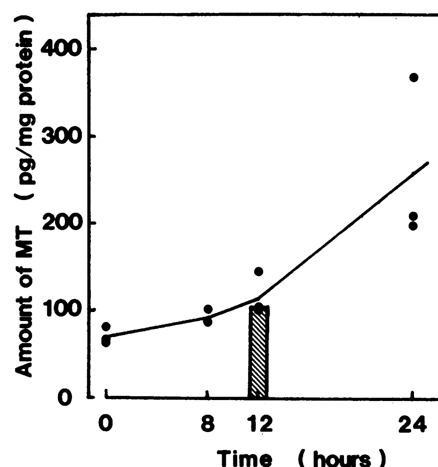

FIG. 5. Increased synthesis of MT in the cells treated with 1α,25(OH)$_2$D$_3$ or dexamethasone. FRSK cells were exposed to 1α,25(OH)$_2$D$_3$ at 5 ng/ml or 10 μM dexamethasone for the indicated period, and amounts of MT were quantitated by RIA using sheep antiserum against rat hepatic MT-I. Points, MT (pg/mg of protein) in separate culture dishes. A line is drawn on mean values. Histogram, MT (pg/mg of protein) of the cells treated with 10 μM dexamethasone for 12 hr.

shown). The major transcript of the MT gene was $\approx$0.5 kb long as observed in FRSK cells. A larger transcript of 2–4 kb seemed to be a precursor of MT mRNA.

DISCUSSION

In the present study, we demonstrated that MT mRNA was induced by 1α,25(OH)$_2$D$_3$ in epidermal keratinocytes *in vitro* and in liver, kidney, and skin tissues *in vivo*. The expression of the MT gene has been shown to be regulated by a wide variety of environmental and physiological substances including heavy metals (31, 32), TPA (33, 34), growth factors (34), UV irradiation (35), lipopolysaccharide (36), interleukin 1 (37), interferon (38), and glucocorticoids (39). But as far as we know, 1α,25(OH)$_2$D$_3$ has not been reported to be a regulator of MT gene expression.

Although 1α,25(OH)$_2$D$_3$ is formed metabolically from the vitamin, it is now regarded as a hormone, because of its presence in the plasma at a certain concentration (40 pg/ml) and because of its action through binding to a cytosol/nuclear receptor (for review, see ref. 40). The skin and cultured epidermal keratinocytes are known to contain a specific receptor for 1α,25(OH)$_2$D$_3$ (for review, see ref. 41). The presence of its receptor in the kidney was also demonstrated (42, 43). There is no report of its receptor in the liver, but we detected them in some established liver cell lines including IAR-20.

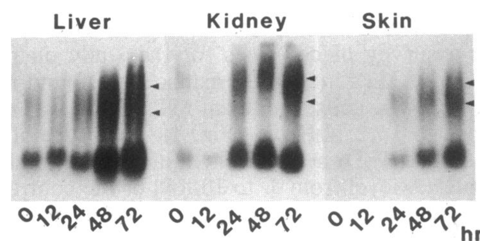

FIG. 6. Induction of MT mRNA in the liver, kidney, and skin of 1α(OH)D$_3$-treated mice. Female Sencar mice were given 1α(OH)D$_3$ at 5 μg per mouse by gastric tube, and their liver, kidney, and skin tissues were removed 12, 24, 48, and 74 hr later. Total cellular RNA was isolated and subjected to RNA gel blotting. Control mice (0 hr) were treated with the solvent (medium-chain triacylglycerol), and RNA was isolated 48 hr later.

$1\alpha,25(OH)_2D_3$ is thus a steroid hormone. Like glucocorticoids, $1\alpha,25(OH)_2D_3$ may regulate transcription of the MT gene by binding to a specific site in the gene. Such regulatory DNA elements have been identified in the 5′-flanking region of the MT gene and also in the long terminal repeat of mouse mammary tumor virus (44–46). A consensus sequence for the binding sites of the glucocorticoid-receptor complex has been proposed on the basis of nucleotide sequence homology (45). It would be of particular interest to identify a specific sequence, if present, to which the $1\alpha,25(OH)_2D_3$-receptor complex binds and so regulates induction. Alternatively, $1\alpha,25(OH)_2D_3$ may regulate expression of the MT gene at a pretranslational level other than transcription, such as half-life of MT mRNA or processing of the mRNA. Further studies are needed on molecular mechanisms of the gene regulation by $1\alpha,25(OH)_2D_3$.

The time courses of MT mRNA induction differed in various experimental conditions. In cultured cells, MT mRNA increased within 2 hr of exposure to $1\alpha,25(OH)_2D_3$ and reached a maximum at 12–24 hr. However, when $1\alpha(OH)D_3$ was administered orally, the induction of MT mRNA in the liver, kidney, and skin was not evident until 24 hr and reached a maximum level at 48–72 hr. This delay in the induction may be explained by the times required for metabolic conversion of $1\alpha(OH)D_3$ to $1\alpha,25(OH)_2D_3$ and its tissue accumulation. Alternatively it is also possible that this delayed induction of MT mRNA was not due to the direct action of $1\alpha,25(OH)_2D_3$ but was a secondary response, e.g., to increase in the plasma level of Ca^{2+}. Indeed, the time course of MT mRNA induction in $1\alpha(OH)D_3$-treated mice coincided with that of hypercalcemia (12). In cultured cells, this possibility still remains, although a secondary response due to expression of another gene was excluded by the combined treatment with $1\alpha,25(OH)_2D_3$ and cycloheximide.

The structure and regulation of the MT gene have been investigated extensively, but the physiological functions of MTs are still under investigation (1). It seems likely that they are in some way involved in a wide range of protections against exogenous stimuli. MTs have been shown to act as free-radical scavengers (2). This function is particularly interesting, because several lines of evidence indicate that tumor promotion and the actions of tumor promoters are attributable to the formation of free radicals (47). The inhibitory effects of $1\alpha,25(OH)_2D_3$ on tumor promotion and on ornithine decarboxylase induced by tumor promoters may be related to activation of the MT gene.

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