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Presence of gene expression of vitamin D receptor and 24-hydroxylase in OK cells

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Abstract

We investigated the gene expression of 1,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$) receptors (VDR) and 25-hydroxy-vitamin D_3-24-hydroxylase (24-hydroxylase) in opossum kidney (OK) cells. Reverse transcription-polymerase chain reaction demonstrated the presence of VDR gene expression in OK cells. Northern hybridization, using rat VDR cDNA, also demonstrated a 4.4 kbp VDR mRNA expression which was affected by serum but not by $1,25(OH)_2D_3$ and/or parathyroid hormone stimulation. Stimulation with $1,25(OH)_2D_3$ induced 24-hydroxylase mRNA expression which peaked at 6 h. This study clearly demonstrates for the first time the presence of VDR and 24-hydroxylase gene expression in OK cells, suggesting that OK cells are a useful model for investigating the genetic action mechanisms of $1,25(OH)_2D_3$.

Key words: OK cell; 1,25-Dihydroxy-vitamin D; Vitamin D receptor; 24-Hydroxylase; Gene expression

1. Introduction

OK cells, which have parathyroid hormone (PTH) receptors, have several features characteristic of proximal tubular cells. Several studies using OK cells have been made on proximal tubular functions, such as Na/P_i co-transport, responsiveness to PTH, amino acid absorption [1–3]. It is not clear, however, whether OK cells preserve biological responsiveness to 1,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$ [3,4] which biologically targets proximal tubular function [5]. Allon and Paris reported that, of the OK cell subclones, only OK-7A subclones have biological responsiveness to PTH in terms of Na/P_i co-transport, while other subclones, including wild-type OK cells, do not respond to PTH [3]. Unresponsiveness to $1,25(OH)_2D_3$ may be due to the lack of or abnormalities in $1,25(OH)_2D_3$ receptors (VDR) [3,4].

The aim of the present study is to examine the presence of VDR gene expression in OK cells (wild-type). An investigation was also made of the effects of $1,25(OH)_2D_3$ and/or PTH on VDR mRNA expression and the effects of $1,25(OH)_2D_3$ on 25-hydroxyvitamin D_3-24-hydroxylase (24-hydroxylase), the latter having been recently demonstrated to have vitamin D responsive elements in the promoter of the gene [6].

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2. Materials and methods

2.1. OK cells and cultures

OK cells (wild-type), an established continuous cell culture from opossum kidneys [7], were kindly provided by Dr. S. Sasaki (Tokyo Medical and Dental University School of Medicine, Tokyo, Japan) at passage no. 72. All experiments were performed with cells from passages 74–76. The cells were cultured with Dulbecco's Modified Essential Medium (DMEM) (Gibco, Grand Island, NY), containing 10% fetal calf serum (FCS) (Gibco) supplemented with 50 μg/ml penicillin and 50 μU/ml streptomycin (Gibco) in a humidified atmosphere of 37°C and 5% CO_2. The cultures were changed to fresh medium every 4 days.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Using 20-mer oligonucleotide primers (5′-GACCGCCTATCCAACACGCT-3′, 5′-GGAGATCTCATTGCCGAACT-3′) derived from rat VDR cDNA sequence [8], RT-PCR was performed to examine the presence of mRNA expression of VDR in OK cell extracts. In the first experiment, total RNA was isolated by acid guanidium thiocyanate-phenol chloroform extraction [9]. In the second experiment, $1,25(OH)_2D_3$ (10^{-7} M) (kindly provided by Chugai Pharmaceuticals Co. Ltd., Tokyo, Japan) was added to each flask when the culture mediums were changed, and total RNA was isolated before incubation and 3, 6, 12 and 24 h after incubation. Reverse transcription (RT) was performed in 10 μl of reaction mixture which contained 1 μg of total RNA extracted from OK cells, 10 nM of each deoxynucleotide triphosphate, 10 pM of random hexamer (Takara Shuzo Co. Ltd., Kyoto, Japan), 20 U of ribonuclease inhibitor (Takara Shuzo Co. Ltd.), and 100 U of MoMuLV-reverse transcriptase (Gibco) in 1 × RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$). Reaction was carried out at 37°C. cDNA was amplified in 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 0.1 mg/ml of gelatin) with 0.1 mM of each deoxynucleotide triphosphate, 0.5 μM of each primer, and 25 U/ml of Taq polymerase (Takara Shuzo Co. Ltd.). Amplification was carried out at 30 cycles for 60 s at 90°C, for 60 s at 55°C, and for 90 s at 72°C, successively. Amplified RT-PCR products were further identified as VDR cDNA by hybridization with rat VDR

cDNA. In brief, RT-PCR products were electrophoresed in 1% agarose gel, and then transferred to a nylon filter (Hybond-N, Amersham International, Buckinghamshire, UK). The filter was hybridized for 40 hours at 37°C with ^{32}P-labelled cDNA probe from rat VDR in 50% formamide, 3 × SCC (0.45 M NaCl, 0.045 M sodium citrate, pH 7.4), 50 mM Tris/HCl pH 7.5, 0.1% sodium dodecyl sulfate, 20 μg/ml tRNA, 20 μg/ml boiled salmon sperm DNA and 1 mM EDTA. cDNA for rat VDR was kindly provided by Dr. H.F. DeLuca [8] and cDNA probe was labelled with [α-^{32}P]dCTP (30,000 Ci/mmol) (Dupont/New England Nuclear, Boston, MA) using the hexadeoxynucleotide random extension method. The filter was exposed to X-ray film (Fuji Film Co. Ltd., Tokyo, Japan).

2.3. Northern hybridization using rat VDR cDNA

OK cells were cultured in 175 cm^2 plastic flasks until they were confluent. In the first experiment, in order to demonstrate VDR gene expression in OK cells, total RNA was extracted by the method described above 4 days after changing the flask medium. In the second experiment, in order to examine the effect of 1,25(OH)$_2$D$_3$ on mRNA expression, either 1,25(OH)$_2$D$_3$ (10^{-7} M) or vehicle (0.01% ethanol at the final concentration) was added to each flask when the culture medium was changed, and total RNA was isolated from each flask before incubation and 3, 6, 12 and 24 h after incubation in the freshly changed medium with 1,25(OH)$_2$D$_3$ or vehicle. In the third experiment, in order to examine the effect of PTH and/or 1,25(OH)$_2$D$_3$, human 1–34PTH (10^{-7} M) (kindly provided by Asahi Kasei Pharmaceutical Co. Ltd., Tokyo, Japan) and/or 1,25(OH)$_2$D$_3$ (10^{-7} M) were added to each flask when the culture medium was changed, and RNA was isolated from each flask 6 h after incubation. In these experiments, after total RNA was isolated by acid guanidium thiocynate-phenol chloroform extraction [9], poly(A)$^+$ RNA was extracted by using oligo(dT)-latex column (Takara Shuzo Co. Ltd.). One μg of the poly(A)$^+$ RNA was electrophoresed in a 1% agarose gel containing formaldehyde, and then transferred to a nylon filter (Hybond-N) and hybridized for 40 h at 37°C with ^{32}P-labelled cDNA probe from rat VDR in the same hybridization solution described above. The filter was exposed to X-ray film.

2.4. Northern hybridization using 24-hydroxylase cDNA

Using 24-hydroxylase cDNA probe, which is a 410 bp fragment cloned from rat duodenum [10–12], Northern hybridization was carried out to examine the effects of 1,25(OH)$_2$D$_3$ on 24-hydroxylase mRNA expression. OK cells were cultured in 175 cm^2 plastic flasks. When the culture medium was changed, either 1,25(OH)$_2$D$_3$ (10^{-7}) or vehicle (0.01% ethanol at final concentration) was added to each flask. Before incubation and 3, 6, 12 and 24 h after incubation with 1,25(OH)$_2$D$_3$ or vehicle, total RNA from each flask was isolated and poly(A)$^+$ RNA was extracted by the method described above. Northern hybridization was carried out by the same procedure described above, except that cDNA probe was used for 24-hydroxylase.

3. Results

3.1. Demonstration of the presence of VDR gene expression

In the first Northern hybridization experiment, in which RNA was isolated from OK cells 4 days after changing the medium, hybridized bands with rat VDR cDNA were too weak to be detected by autoradiography (0 hour lane of Fig. 1). It was therefore decided to perform RT-PCR in order to demonstrate the possible presence of VDR gene expression in OK cells. The ultraviolet fluorogram of an agarose gel electrophoresis of RT-PCR products stained with ethidium bromide clearly demonstrated a single band at 213 bp (data not shown). Electrophoresised RT-PCR product was transferred to a nylon membrane and hybridization was performed with rat VDR cDNA probe. A single band of very strong intensity at 213 bp was clearly observed (0 hour lane of Fig. 2).

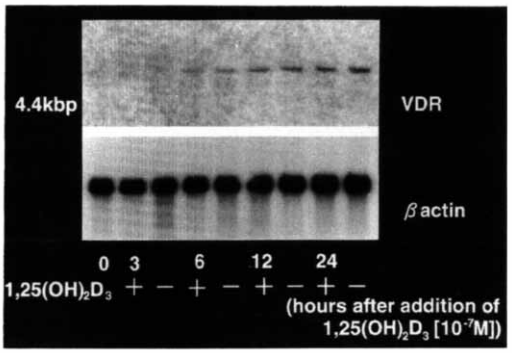

Fig. 1. Northern hybridization with cDNA for rat VDR and β-actin. In the left lane, RNA was extracted from OK cells which were cultured for 4 days (0 hour). In remaining lanes, RNA was extracted from OK cells 3, 6, 12 and 24 h after incubation in a fresh medium with 1,25(OH)$_2$D$_3$ or vehicle. Similar results were obtained in three independent sets of experiments.

3.2. Effects of 1,25(OH)$_2$D$_3$ and/or PTH on VDR mRNA expression

In the second RT-PCR experiment, in which OK cells were incubated with 1,25(OH)$_2$D$_3$, intensity of the hybridized bands was higher in the lane for RT-PCR products from OK cells that had had a longer incubation period in a fresh medium with 1,25(OH)$_2$D$_3$ (Fig. 2). RT-PCR did not allow accurate estimation of the amount of VDR mRNA expression, due to the fact that amplification was only carried out at 30 cycles. In the second Northern hybridization experiment, in which OK cells were incubated in fresh medium either with or without 1,25(OH)$_2$D$_3$, a single hybridized band with rat VDR cDNA was observed at 4.4 kbp in certain lanes, though the intensity of the band was too weak in other lanes to be detected by autoradiography. Time course of VDR gene expression after stimulation with 1,25(OH)$_2$D$_3$ or vehicle (0.01% ethanol) is shown in Fig. 1. VDR gene expression increased after incubation in fresh media with either 1,25(OH)$_2$D$_3$ or vehicle. These results indicate the stimulatory effects of changing to a fresh medium but do not indicate the effects of 1,25(OH)$_2$D$_3$ itself on VDR gene expression. Fig. 3 shows the result of the third Northern hybridization experiment in which OK cells were incubated in freshly changed medium for 6 h with 1,25(OH)$_2$D$_3$ and/or PTH or vehicle. A single band was again observed at 4.4 kbp in each lane. There was little specific effect of PTH or 1,25(OH)$_2$D$_3$ on VDR mRNA expression when VDR gene expression intensity in each band was corrected by β-actin.

3.3. Results of Northern hybridization with 24-hydroxylase cDNA

Fig. 4 shows the result of Northern hybridization using 24-hydroxylase cDNA. Although gene expression

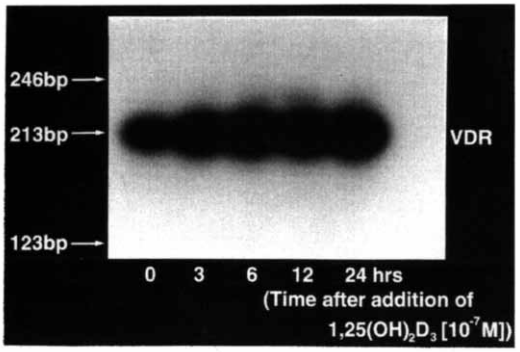

Fig. 2. Hybridization of RT-PCR products with rat VDR cDNA. In the left lane, RNA was extracted from OK cells which were cultured for 4 days (0 hour). In the remaining lanes, RNA was extracted from OK cells 3, 6, 12 and 24 h after incubation in a fresh medium with $1,25(OH)_2D_3$.

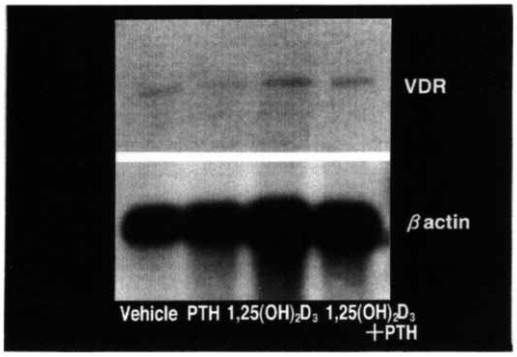

Fig. 4. Northern hybridization with 24-hydroxylase cDNA. RNA was extracted from OK cells before incubation and 3, 6, 12 and 24 h after incubation in a fresh medium with $1,25(OH)_2D_3$ or vehicle. Similar results were obtained in three independent sets of experiments.

was not detected before stimulation with $1,25(OH)_2D_3$ or after incubation with vehicle (0.01% ethanol), a single band was observed at 3.4 kbp in the lanes of the OK cell samples stimulated with $1,25(OH)_2D_3$. The 3.4 kbp gene expression corresponds to the size of the gene expression reported previously [12–14]. After $1,25(OH)_2D_3$ stimulation, gene expression peak was seen at 6 hours, while faint expression was observed even after 24 h.

4. Discussion

OK cells have several features characteristic of proximal tubular cells, which are target cells for $1,25(OH)_2D_3$ [5]. It is not clear, however, whether OK cells have biological responsiveness to $1,25(OH)_2D_3$ [3,4]. Allon and Parris reported that, though ligand binding assay revealed VDR in all the OK cell subclones, only OK-7A subclones are biologically responsive to $1,25(OH)_2D_3$ in terms of Na^+/P_i co-transport, which suggests VDR abnormalities in other subclones [3]. It is not known, however, whether OK cells, such as the wild-type OK cells used in the present study, are genetically responsive to

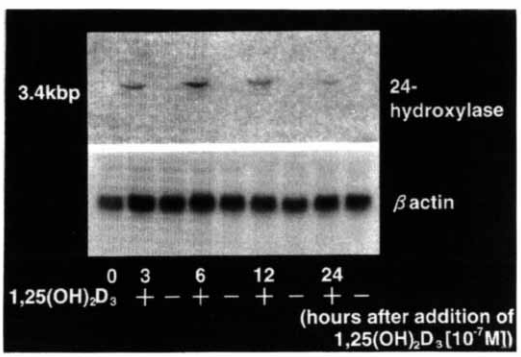

Fig. 3. Northern hybridization with cDNA for rat VDR and β-actin. RNA was extracted from OK cells 6 h after incubation in a fresh medium with vehicle or PTH and/or $1,25(OH)_2D_3$. Similar results were obtained in three independent sets of experiments.

$1,25(OH)_2D_3$. Nor have there been any reports so far on VDR gene expression.

Northern hybridization with rat VDR cDNA did not allow clear detection of the presence of VDR mRNA in OK cells which had been cultured for 4 days in the medium (0 hour lane of Fig. 1). RT-PCR, however, did demonstrate the presence of VDR mRNA expression in the cells. The RT-PCR products were identified as VDR cDNA using a hybridization with rat VDR cDNA (0 hour lane of Fig. 2). Furthermore, using rat VDR cDNA, another Northern hybridization of $poly(A)^+$ RNA from OK cells that were cultured in a fresh medium demonstrated a band at 4.4 kbp in some lanes (Fig. 1), the size being identical to that reported previously [12–14]. Through these experiments, the presence of VDR gene expression in OK cells was demonstrated for the first time.

In the RT-PCR experiments, VDR mRNA expression seemed to be higher in OK cells that had been incubated for a longer period in a fresh medium with $1,25(OH)_2D_3$. However, due to the fact that the amplification was only carried out at 30 cycles, RT-PCR in the present study is not suitable for assessing the amount of VDR mRNA expression in OK cells. Using rat VDR cDNA in Northern hybridization in which OK cells were stimulated with $1,25(OH)_2D_3$ or vehicle, the amount of VDR gene expression increased by stimulation with either $1,25(OH)_2D_3$ or vehicle in fresh medium. This result suggests that a fresh medium containing fresh FCS, rather than $1,25(OH)_2D_3$, stimulated the VDR mRNA expression. Stimulatory effects of FCS on VDR gene expression in culture cells has also been reported by Krishnan and Feldman [15]. VDR protein has been reported to be up-regulated by $1,25(OH)_2D_3$ in both in vitro and in vivo experiments on various cells [16–20], while VDR gene regulation by $1,25(OH)_2D_3$ varies from cell to cell. A recent study from this laboratory has demonstrated that VDR gene expression does not correlate to the amount of VDR protein in rat intestine, showing that, after

$1,25(OH)_2D_3$ stimulation, VDR gene expression was not increased but that VDR protein was [21]. Wiese et al. also suggest that VDR molecules are regulated by $1,25(OH)_2D_3$ at a post-transcriptional or translational level [16]. In other reports, however, VDR genes have been upregulated by using $1,25(OH)_2D_3$ in parathyroid cells and osteoblast-like cells [22,23]. So far there has been no report on cultured renal tubular cells addressing VDR gene regulation by $1,25(OH)_2D_3$. The present study shows that $1,25(OH)_2D_3$ does not affect the expression of VDR mRNA in OK cells. Furthermore, in the third Northern hybridization study, in which OK cells were incubated with PTH and/or $1,25(OH)_2D_3$, little specific effect of PTH on VDR mRNA expression seemed to be observed. These results do not coincide with those of other reports in which PTH in vivo down-regulated VDR mRNA expression in the kidney and intestine [23,24]. Since Northern hybridization was not performed enough times for statistical analysis, conclusions as to the effects of PTH will not be elucidated in this particular report.

In the Northern hybridization experiments using 24-hydroxylase cDNA, 24-hydroxylase mRNA expression was not detected before $1,25(OH)_2D_3$ stimulation or after vehicle stimulation. Gene expression was induced by $1,25(OH)_2D_3$ as early as 3 h, with its peak at 6 h. In vivo rat studies, 24-hydroxylase genes were rapidly induced by administering $1,25(OH)_2D_3$ within one hour after treatment [10,11,14,24]. These reports suggest that 24-hydroxylase gene expression is regulated by $1,25(OH)_2D_3$ by a *cis*-regulatory mechanism. Recent studies from this laboratory have shown protein kinase C involvement in 24-hydroxylase gene expression that has been induced in rat intestinal epithelial cell cultures by $1,25(OH)_2D_3$ [10,11]. So far, neither 24-hydroxylase activity nor its gene expression have been found in cell cultures, such as OK cells, derived from kidneys. The present study indicate that OK cells may be useful for analysing the genetic action mechanisms of $1,25(OH)_2D_3$ on tubular cells, in terms of early induction of 24-hydroxylase genes.

In conclusion, the present study clearly demonstrates for the first time the presence of VDR gene expression in OK cells, and that VDR gene expression does not seem to be affected by $1,25(OH)_2D_3$ or PTH. Furthermore, OK cells, when treated with $1,25(OH)_2D_3$, show 24-hydroxylase gene expression. OK cells could be a useful model of renal cell cultures in the analysis of the genetic action mechanisms of $1,25(OH)_2D_3$.

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