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GROWTH INHIBITION OF HUMAN KERATINOCYTES BY 1,25-DIHYDROXYVITAMIN D_3 IS LINKED TO DEPHOSPHORYLATION OF RETINOBLASTOMA GENE PRODUCT

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Human keratinocyte is one of the target cells for $1,25(OH)_2D_3$, a biologically active form of vitamin D_3. It induces the differentiation and growth inhibition of human keratinocytes. In order to understand the inhibitory mechanism of $1,25(OH)_2D_3$, we examined its effect on cell cycle kinetics and retinoblastoma gene product (pRB), one of tumor suppressor gene products, in normal human keratinocytes. Cell cycle analysis demonstrated that 10^{-6} M of $1,25(OH)_2D_3$ induced cell cycle arrest in both G1/G0 (63.4 % ± 1.4 versus 52.7% ± 1.2 in control, $p< 0.05$) and G2+M (21.5%± 0.6 versus 10.9% ± 0.8 in control, $P< 0.05$) phase. Addition of 10^{-6}M of $1,25(OH)_2D_3$ increased dephosphorylated pRB in a time dependent manner from 23% at 0 h to 58% at 48 h. Since the phosphorylation of pRB is supposed to be essential for the progression from G1 to S phase, the inhibition of pRB phosphorylation could be responsible for the G1/G0 growth arrest induced by $1,25(OH)_2D_3$ in normal human keratinocytes .

Accumulation of the studies on vitamin D_3 revealed that 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], an active form of vitamin D_3, modulates various biological functions in a variety of cells . $1,25(OH)_2D_3$ exerts its effects via binding to an intracellular receptor which is a member of the steroid, thyroid, and retinoic acid receptor gene family [1,2]. The presence of vitamin D_3 receptor was confirmed in many kinds of cells and tissues [3]. Recently, the role of vitamin D_3 as an antiproliferative agent has attracted much attention in a variety of cells such as human leukemic cell line [4], breast cancer cell line [5] and human keratinocytes [6]. These studies led to the exploration for the possibility of vitamin D_3 as a new therapeutic agent. Among them, the most successful application of vitamin D_3 as an antiproliferative drug is the treatment of psoriasis, a skin disease characterized by hyperproliferation of epidermal keratinocytes [7].

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The effect of $1,25(OH)_2D_3$ on keratinocytes has been well characterized. Hosomi et al. [8] demonstrated that $1,25(OH)_2D_3$ facilitates terminal differentiation of cultured mouse epidermal cells, and Smith et al. [9] reported the enhancement of morphological differentiation of human keratinocytes by $1,25(OH)_2D_3$. Matsumoto et al. showed that it inhibits human keratinocyte growth [6]. However, the mode of antiproliferative action of $1,25(OH)_2D_3$ has not been fully clarified in keratinocytes. The retinoblastoma gene product (pRB) is a 105 kilodalton nuclear protein. Its gene was first discovered as a gene in which mutational inactivation resulted in the development of ocular tumors in children [10]. Then, it has been shown that pRB is a tumor suppressor [11,12]. Besides the character of a tumor suppressor, pRB is also involved in the growth regulation of normal cells, namely cell cycle regulation. It undergoes cell-cycle-dependent phosphorylation and dephosphorylation [13-15]. The pRB is dephosphorylated in G1 phase and becomes multiple-phosphorylated near the G1/S boundary. And it remains highly phosphorylated through G2 and early M. Then pRB is dephosphorylated as the cell moves into late M or early G1 phase [13-16]. Therefore, conversion between phosphorylated and dephosphorylated pRB is one of the crucial steps in cell cycle regulation.

To elucidate the mechanism of vitamin D_3-induced growth inhibition, we analyzed cell cycle distribution in human keratinocytes treated with $1,25(OH)_2D_3$ and the alteration of phosphorylated and dephosphorylated state of pRB. In this paper, we showed that $1,25(OH)_2D_3$ induced growth arrest in G1/G0 and G2+M and the increase of the dephosphorylated form of pRB.

Materials and Methods

Cell culture: Normal human skin samples were obtained during plastic surgery. Human keratinocytes were cultured as described previously [17]. Briefly, skin was cut into 3 to 5 mm square pieces, and floated on dispase solution (500 U/ml) overnight at 4 °C. After separation of epidermis from dermis by forceps, the epidermal sheets were rinsed with Ca^{2+} and Mg^{2+}-free PBS and incubated in 0.25% trypsin solution for 10 min at 37 °C, and then the epidermis was teased with forceps. Cells were suspended and cultured in MCDB 153 medium (Kyokuto Co., Tokyo, Japan) supplemented with insulin (5 μg/ml), hydrocortisone (5×10^{-7} M), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM) and bovine hypothalamic extract (150 μg/ml). EGF was not added. The stock solution consisted of MCDB 153 supplemented with 0.1 mM Ca^{2+} plus extra amino acids (0.75 mM isoleucine, 0.24 mM histidine, 0.09 mM methionine, 0.09 mM phenylalanine, 0.045 mM tryptophan and 0.075 mM tyrosine) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). Second-passage cells were used in this experiment.

Cell cycle analysis: The cell cycle analysis of human keratinocytes were performed by the two color cell cycle analysis method described by Dolbeare et al [18]. Briefly, subconfluent keratinocytes were cultured in the absence or presence of 10^{-8} or 10^{-6}M $1,25(OH)_2D_3$ for 48 h. To further study the early change of cell cycle in detail, we analyzed it every 3 h for 30 h in the presence of 10^{-6}M $1,25(OH)_2D_3$. And pulse-labeled with 0.01 mM 5-bromodeoxyuridine (BrdU) for 120 min. BrdU

incorporated into the nucleus was immunochemically stained using anti-BrdU monoclonal antibody (Becton Dickson Immunocytometry Systems, San Jose, CA), and DNA was stained with propidium iodide. These stained cells were analyzed by flow cytometry using FACScan (Becton Dickson Immunocytometry Systems, San Jose, USA).

Analysis of pRB phosphorylation: The analysis was performed according to the method obtained from the supplier of monoclonal antibodies to pRB (Pharmingen, San Diego, USA). Keratinocytes were grown on 3.5 cm dishes. The cells were washed with 3 ml of phosphate buffered saline (PBS) and solubilized with 200 µl of lysis buffer (1% SDS, 0.08 M Tris, 10% glycerol, DTT 15.4 mg/ml, aprotinin 50 µg/ml, leupeptin 21.2 µg/ml, E-64 10 µg/ml). The dishes were shaken gently on ice for 15 min. The lysates were centrifuged and the supernatant was removed to a clean tube, and boiled for 5 min. The samples (40 µg protein on each lane) were applied to 7.5% polyacrylamide gels containing 0.1% SDS. The resolved proteins were transferred electrophoretically to PVDF (Polyvinylidene difluoride) membrane at 180 mA for 60 min. The membrane was washed once with TBST (0.025 M Tris, 0.125 M NaCl, 0.025% Tween 20, pH7.2) for 10 min. Then the blot was shaken for one hour at 4°C in blocking buffer (5% dry non-fat milk, 0.05% Tween 20 and 0.1% NaN_3), followed by incubation for 60 min at 4°C in the same buffer containing 10 µg/ml anti-pRB monoclonal antibody (Mh-Rb-02 P, Pharmingen, San Diego, USA). The blot was rinsed three times in TBST for 15 min at 4°C, incubated in 1% BSA/TBST with 1:500 dilution of alkaline-phosphatase-conjugated goat anti-mouse IgG (Problot System, Promega, Madison USA) at room temperature for 60 min, and washed three times in TBST at room temperature for 15 min. The Histfine First Blue (Nitirei, Tokyo, Japan) was then used for visualization of alkaline phosphatase by incubating for 5 min at room temperature. The reaction was stopped by washing with water. The diffuse 114-116 kilodalton bands were detected as phosphorylated pRB. In contrast, the 105 kilodalton band was detected as dephosphorylated pRB. Bands on the Western blots were quantified by densitometer (CS-9000, Shimadzu, Kyoto, Japan). The percentage of dephosphorylated pRB to total pRB was calculated based on densitometric data.

Results

Effect of $1,25(OH)_2D_3$ on cell cycle of keratinocytes: In order to investigate the effect of $1,25(OH)_2D_3$ on the cell cycle of keratinocytes, we analyzed cell cycle distribution in cells treated with 10^{-8} M and 10^{-6}M of $1,25(OH)_2D_3$. Cells were cultured in the absence or presence of this compound for 48 h, and then cell cycle distribution was analyzed by flow cytometry. Cell cycle distribution in these cells were calculated from contour graphs. As shown in table 1, 10^{-8} M of $1,25(OH)_2D_3$ had no effect on cell cycle, but 10^{-6}M of $1,25(OH)_2D_3$ decreased the cell number in S phase from 36.4 ± 1.8% to 15.2 ± 0.7%. In contrast, it increased the cell number in G1/G0 and G2+M phases from 52.7 ± 1.2% to 63.4 ± 1.4% and from 10.9 ± 0.8% to 21.5 ± 0.6%, respectively. These observation is consistent with the previous report on growth inhibition [6] since the growth inhibition is marked at 10^{-6}M but not at 10^{-8} M. Next, we examined cell cycle distribution in time course after treatment with $1,25(OH)_2D_3$. Cells were cultured in the presence of 10^{-6}M $1,25(OH)_2D_3$ and cell

Table1
Percentage cell cycle distribution in control and $1,25(OH)_2D_3$ treated cultures of keratinocytes

	G1/G0	S	G2+M
Control	52.7 ± 1.2	36.4 ± 1.8	10.9 ± 0.8
$1,25(OH)_2D_3$ 10^{-8}M	54.0 ± 2.4	35.2 ± 2.5	10.8 ± 0.4
10^{-6}M	63.4* ± 1.4	15.2* ± 0.7	21.5* ± 0.6

Cell cycle distribution was analyzed by flow cytometry, using a FACScan. Cells were harvested at 48 h after treatment with $1,25(OH)_2D_3$. Values represent the means and SD of triplicate measurements. Asterisks show the statistically significant values ($p<0.01$). p value was calculated using the Student t test.

cycle distribution was measured every 3 h up to 30 h (Fig 1). The cell number in S phase decreased at as early as 3 h. It reached the lowest, 8.8% at 18 h and the plateau after 18 h. In contrast, the cell number in G1/G0 increased from 49.9% at 0 h to 67% at 18 h maximally. This indicates that G1/G0 arrest is mainly responsible for the decrease of S phase cells. The G1/G0 cells showed a temporary decline at 3 h. This appears to be due to the delay of transit from G2 + M cells to G1/G0, since the cells in G2 + M phase increased from 15. 4% at 0 h to 22.5% at 3 h.

Effect of $1,25(OH)_2D_3$ on retinoblastoma gene product (pRB): Addition of 10^{-6}M of $1,25(OH)_2D_3$ induced the increase of dephosphorylated pRB and the decrease of

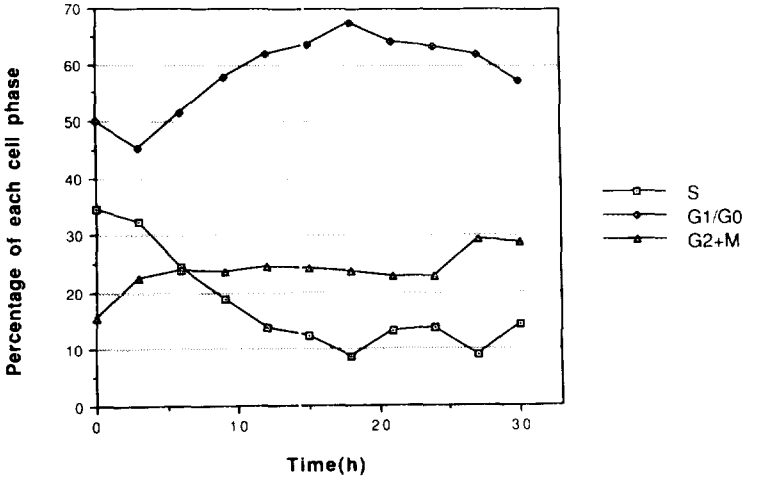

Figure 1.
Time course of the effect of $1,25(OH)_2D_3$ on cell cycle . Cells were harvested at 3 h intervals up to 30 h after treatment with 10^{-6}M $1,25(OH)_2D_3$.

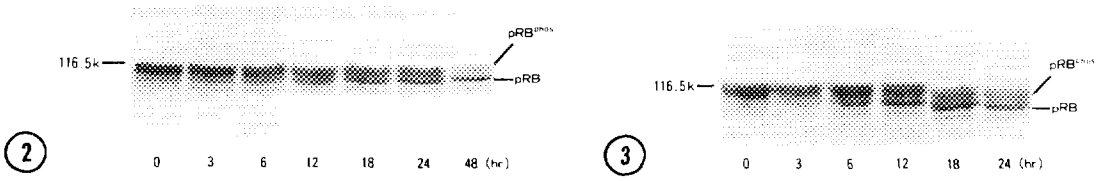

Figure 2.
Conversion of phosphorylated pRB to dephosphorylated pRB by $1,25(OH)_2D_3$. Cells were harvested at 0 h (lane 1), 3 h (lane 2), 6 h (lane 3), 12 h (lane 4), 18 h (lane 5), 24 h (lane 6) and 48 h (lane 7) after treatment with 10^{-6}M $1,25(OH)_2D_3$. The phosphorylated and dephosphorylated pRB were analyzed by Western blot as described under *Materials and Methods*. pRB^{phos} and pRB indicate phosphorylated and dephosphorylated pRB, respectively. Molecular weight for phosphorylated pRB (116.5 k) is shown on the left side.

Figure 3.
Conversion of phosphorylated pRB to dephosphorylated pRB by TGF-ß1. Cells were harvested at 0 h (lane 1), 3 h (lane 2), 6 h (lane 3), 12 h (lane 4), 18 h (lane 5) and 24 h (lane 6) after treatment with 10 ng/ml TGF-ß1.

phosphorylated one (Fig 2). The increase of dephosphorylated pRB band was observed as early as at 6 h, and reached maximum at 18 h. This pattern is almost inversely proportional to the number of S phase cells. In contrast, the phosphorylated pRB bands turned wider and more faint. Densitometer measurement indicated that the percentage of dephosphorylated pRB increased from 23% at 0 h to 58% at 48 h. Since TGF-ß1 was reported to increase the dephosphorylated pRB in human keratinocytes [19], we also examined the effect of TGF-ß1 on pRB (Fig 3). Addition of TGF-ß1 at 10 ng/ml caused almost the similar change of pRB in human keratinocytes, although the conversion of phosphorylated to dephosphorylated pRB was almost complete at 24 h. This could be dependent on the concentration of TGF-ß1.

Discussion

We have already reported on the characterization of antiproliferative potential by $1,25(OH)_2D_3$ in human keratinocytes [6]; 1) the rapid inhibition of DNA synthesis; 2) the decrease in the number of high-affinity receptors for epidermal growth factor; 3) the rapid decrease in c-myc mRNA level. In this paper, we further studied the effect of $1,25(OH)_2D_3$ on the cell cycle kinetics and pRB. First, $1,25(OH)_2D_3$ at 10^{-6} M caused growth arrest of human keratinocytes in both the G1/G0 and G2 + M phases of the cell cycle. Then, we showed the dephosphorylation of retinoblastoma gene product (pRB) by $1,25(OH)_2D_3$. The increase of dephosphorylated pRB band occurred rather at early stage. And this increase is almost inversely proportional to the cell number in S phase. Interestingly, we found the rapid decrease of c-myc mRNA at 3 h in the previous report [6]. Taken together, it was suggested that $1,25(OH)_2D_3$ causes the decrease of c-myc and the dephosphorylation of pRB, resulting in growth arrest in G1/G0 [19].

Recently, it has been reported that $1,25(OH)_2D_3$ enhanced antiproliferative effect of TGF-ß1 on cultured human keratinocytes and increased the TGF-ß1 mRNA approximately 5 fold [20]. Further, when keratinocyte cultures were treated with $1,25(OH)_2D_3$ in the presence of neutralizing antibodies to TGF-ß, the antiproliferative activity by vitamin D_3 was blocked to some extent. These data indicate the possibility that antiproliferative action by $1,25(OH)_2D_3$ is mediated by TGF-ß1, at least partially. TGF-ß1 is known to inhibit phosphorylation of pRB in rat liver epithelial cell line [16], mink lung epithelial cell [21] and human keratinocytes [19]. Therefore, we compared TGF-ß1 and $1,25(OH)_2D_3$ in regard to the ability to induce the dephosphorylation of pRB. They caused the almost similar change of pRB. This data may support the hypothesis that the effect of $1,25(OH)_2D_3$ is at least partially mediated by TGF-ß.

We also showed accumulation of cells in G2 + M phase by $1,25(OH)_2D_3$. This phenomenon has already reported in breast cancer cell line [5]. We also observed G2 + M arrest of human keratinocytes when differentiation is induced by high Ca^{++} medium (unpublished data). This G2 + M accumulation was also reported when ultraviolet irradiation in combination with psolaren was given [22]. In general, this G2 + M arrest was not remarkable. This is probably the reason why much attention was not payed to this phenomenon. Although the mechanism of G2+M arrest has not been well clarified, the study on G2 + M will also be important to understand the antiproliferative action mechanism by $1,25(OH)_2D_3$.

Our findings provide a background for further studies on the mechanism of $1,25(OH)_2D_3$ action as an antiproliferative agent.

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