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1,25-Dihydroxyvitamin D3 and fetal lung maturation: immunogold detection of VDR expression in pneumocytes type II cells and effect on fructose 1,6 bisphosphatase☆

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Abstract

Lung maturation before birth includes type II pneumocyte differentiation with progressive disappearance of glycogen content and onset of surfactant synthesis.

We have shown previously that 1,25-(OH)2D3 increases surfactant synthesis and secretion by type II cells and decreases their glycogen content in fetal rat lung explants.

Recently, the gene coding fructose 1,6 bisphosphatase (F1,6BP), a regulatory enzyme of gluconeogenesis, has been identified in type II cells and its promoter bears a Vitamin D response element.

Present results show:

1. The coexistence of type II cells at different stages of maturation. in rat fetal lung on day 21 of gestation (electron microscopy), and the association between maturation of type II cells and disappearance of their glycogen content.
2. The immunogold labeling of all type II cells when using the 9A7g VDR-antibody, with significantly more abundant gold particles in cells exhibiting an intermediate glycogen content.
3. The expression of F1,6BP mRNA in a human type II cell line (NCI-H441) and the increase of this expression after 18 h incubation with 1,25-(OH)2D3 (10^{-8} M).

These results bring further evidence for a physiological role of 1,25-(OH)2D3 during type II pneumocyte maturation. Activation of F1,6BP may participate to the 1,25-(OH)2D3 action on surfactant synthesis via the gluconeogenesis pathway.

Keywords: Fetal lung; Glycogen; VDR,1,25-(OH)2D3; Fructose1,6 bisphosphatase

1. Introduction

Functional lung maturation is a process involving type II pneumocyte differentiation. It includes progressive disappearance of the cell glycogen content, and onset of surfactant synthesis (appearance of osmiophilic lamellar bodies, OLBs) and secretion (release of OLBs in the luminal spaces through the apical microvilli).

Our previous studies in fetal rat lung explants suggest that 1,25-(OH)2D3 plays a specific role in lung maturation. This hypothesis stems from several lines of evidence (Table 1):

1. specific binding sites for 1,25-(OH)2D3 (VDR) have been evidenced in fetal rat lung at a time corresponding to the start of type II pneumocyte differentiation and onset of surfactant secretion [1];
2. with use of a monoclonal antibody directed against 1,25-(OH)2D3 receptors, these receptors have been localized to type II pneumocytes [2];
3. in explanted fetal rat lung tissue, 1,25-(OH)2D3 accelerates the decrease in type II cells glycogen content and increases surfactant synthesis (increase in cell

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Table 1
Reported effects of 1,25-(OH)2D3 on rat fetal lung maturation

	Day of gestation (+ day of culture)			
	19 + 1		19 + 2	
+1,25-(OH)2D3 (1 nM)	0	+	0	+
Type II cells (% total epithelial cells)	10 ± 0.1	11 ± 0.2	32 ± 0.15[a]	34 ± 0.2[a]
Apical microvilli (per type II cell)	6.6 ± 0.6	9.1 ± 0.6	11.9 ± 1.4[a]	13.3 ± 0.8[a]
Glycogen content (% total cell surface)	28 ± 0.2	23 ± 0.2	10 ± 0.3[a]	0.01 ± 0.01[a,**]
OLB surface area (mm^2/cell)	2.2 ± 0.2	2.8 ± 0.1	3.5 ± 0.1	3.0 ± 0.3
Intraluminal surfactant (% of cell section)	ne	ne	1.2 ± 0.1	2.2 ± 0.2[**]
Phosphatidylcholine (μg inorganic phosphorus/mg protein)	ne	ne	7.5 ± 0.4	9.5 ± 0.4[**]
Phosphatidylglycerol (μg inorganic phosphorus/mg protein)	ne	ne	0.38 ± 0.03	0.70 ± 0.05[**]

Summary of our reported results on the effects of 1,25-(OH)2D3 in rat fetal lung (see [3,4]); Values are mean ± S.E. of 6–9 explants isolated from 3 separate experiments taken on day 19 of pregnancy . For each explant, measurements on 10-14 cell profiles from two different blocks were averaged.; Explants were cultured in control conditions or in the presence of 1,25-(OH)2D3 (10^{-9} M) for 1 or 2 days; ne: not explored.

[a] Different from day 19 + 1, $P < 0.01$, in the same group.

[**] Different from control, $P < 0.01$, on the same day.

phosphatidylcholine and phosphatidylglycerol contents) and surfactant secretion (migration of newly formed OLBs toward the apex of the cell and extrusion in alveolar lumina) [3,4].

4. a paracrine system for 1,25-(OH)2D3 is present in rat fetal lung and functions during the last 3 days of pregnancy. During this limited period, lung fibroblasts can produce 1,25-(OH)2D3 whereas the adjacent type II cells bear significant numbers of VDR and respond to the hormone by increasing surfactant synthesis and secretion [2].

Recently, the gene coding fructose 1,6 bisphosphatase (F1,6BP), a regulatory enzyme of gluconeogenesis, has been cloned in human lung and identified in type II cells [5,6]. The lung isoenzyme presents high homology with the isoenzyme expressed in liver, kidney and monocytes. Besides its effect on gluconeogenesis, and possibly glycogen synthesis, it has been hypothesized that the lung enzyme may participate in surfactant synthesis via an increase in glucose-6 phosphate (6P) and NADPH synthesis.

Of interest, a Vitamin D response element has been identified in the F1,6BP gene [7] and 1,25-(OH)2D3 induces this gene expression in human differentiated monocytes [8].

The present work had a double aim:

1. To approach the physiological role of 1,25-(OH)2D3 in fetal lung by studying the association between VDR expression (electronimmunogold labeling) and glycogen content of type II cells in rat fetal lungs. Day 21 of pregnancy was chosen for this transversal analysis as the different Type II cell differentiation states coexist at this stage of lung development [9].
2. To test the effect of 1,25-(OH)2D3 on F1,6-BP gene expression in an established human type II cell line, NCI-H441, a cell line which synthesizes specific surfactant phospholipids [10].

2. Materials and methods

2.1. For the ultrastructural studies

Pregnant Sprague-Dawley rats of known gestational age were purchased from Charles River (France). On day 21 of gestation, pregnant mothers were anesthetized, their fetuses were recovered by cesarean section and their lungs were dissected out.

Lung samples were fixed in 4% paraformaldehyde and embedded without decalcification at −50 °C in Lowicryl HM-20 resin using a cryosubstitution Automat (AFS-Leica). Ultrathin sections, collected on collodion-coated Ni grids, were treated with 0.1 M glycine, and then with 10% BSA to block non-specific binding sites. Sections were incubated overnight at +4 °C with the monoclonal antibody 9A7gamma directed against the Vitamin D receptor (VDR), and thereafter with antimouse rabbit immunoglobulin (Zymed). They were then treated with a 15 nm protein G-gold complex, rinsed, postfixed with 2% glutaraldehyde and 1% osmic acid and stained. Negative controls were performed with sections labeling in the absence of the first antibody 9A7g. Sections were examined with a Siemens (Elmiscope 101) transmission electron microscope at 80 KV.

2.2. For the biochemical studies of NCl-H441 responses to 1,25-(OH)2D3

2.2.1. VDR binding study

The human NCI-H441 type II cell line was cultured in RPMI +10% FCS. At confluency, cells were incubated for 16 h in serum-free RPMI with either solvent (ethanol) or 1,25-(OH)2D3 (10^{-8} M; a gift from Roche Laboratory, Basel, Switzerland). The medium then was removed and fresh serum-free RPMI was added to the cells for 1 h. Next, cells were harvested and washed twice with PBS by centrifugation at 2000 × g for 5 min and their cytosols were prepared as described previously. For ligand binding,

cytosols were diluted to a protein concentration of 1 mg/ml with the high salt buffer supplemented with aprotinin (0.4 trypsin inhibitor unit per ml of buffer; Sigma–Aldrich, St Quentin-Fallavier, France). They were incubated for 1 h at +25 °C with 3H 1,25-(OH)2D3 (0.3 nM; specific activity, 160 Ci/mmol, Amersham) in the presence or absence of a 50-fold excess of radioinert hormone. At the end of incubations, bound and free steroids were separated by hydroxylapatite (Bio-Rad Laboratories).

2.2.2. Fructose 1,6-BP gene expression study

At confluency, cells were incubated for 18 h in serum-free RPMI with 1,25-(OH)2D3 (10^{-12} to 10^{-8} M) or its solvent. At the end of the incubation, the medium was removed and cells were rinsed twice with sterile PBS before RNA extraction by the method of Chomczynski and Sacchi [11].

Samples of total RNA (30 μg) were fractionated on 1.2% formaldehyde agarose gels and transferred to nitrocellulose membrane for Northern blot analysis. F1,6-BP mRNA levels were measured by hybridization with a ^{32}P F1,6-BP cDNA probe labeled by the random primer method (Rediprime). The F1,6-BP cDNA (700 pb) was cloned in the Puc 9 plasmid (2300 pb). Filters were stripped and rehybridized to a control probe (G3PDH cDNA). Autoradiographs of the Northern blots were assessed by scanning densitometry. The amounts of F1,6-BP mRNA were normalized to G3PDH to adjust for differences in loading.

3. Results

3.1. VDR immunogold labeling in rat fetal lung

Rat fetal lung on day 21 of pregnancy includes different cell populations besides type II pneumocytes, namely lung fibroblasts, endothelial cells, and some type I pneumocytes. Several type II cell populations coexist with different stages of maturation. In these populations, the cell surface occupied by glycogen varies from a mean value of 30%, in the immature cells, down to an almost null surface, at the latest stage of differentiation (Table 2).

After incubation with the monoclonal 9A7 gamma VDR antibody, ultrathin lung cryosections show an immunogold labeling restricted to the cells with type II characteristics. The VDR labeling is observed in the nucleus, but also in the cytoplasm and endoplasmic reticulum (Fig. 1).

Table 2
Association study between VDR expression and type II cell maturation by immunogold labeling

State of type II cell maturation	Glycogen (% cytoplasm surface)	Anti-VDR-linked gold particles
I	30	19.3 ± 0.4
II	9–16	29.5 ± 1.1; $P < 0.01$
III	0	13.0 ± 0.4

*V*alues are mean ± S.E. of 10–15 different cell determinations.

As shown on Table 2, anti-VDR-linked gold particles are significantly more abundant in cells exhibiting an intermediate glycogen content (state II), as compared to immature type II cells with a high glycogen content (state I), and to more differentiated type II cells (state III).

3.2. 1,25-(OH)2D3 responses of a human pneumocyte type II cell line (NCI-H441)

Human pneumocyte type II cells show a high 1,25-(OH)2D3 specific binding capacity (135 ± 3 fmol/mg proteins). Incubation of the cells for 18 h with 10^{-8} M 1,25-(OH)2D3 led to a 1.4 fold increase in the ability of their cytosol to bind 1,25-(OH)2D3 (186 ± 2 fmol/mg proteins).

In basal culture conditions, F1,6BP mRNA is expressed as a single band of 1.7 kb in this human cell line. Dose-response studies of the effects of a 18 h incubation with 1,25-(OH)2D3 show no effect at the lowest concentrations tested (10^{-12} to 10^{-10} M) , but a 1.25-fold increase with 10^{-8} M (Fig. 2).

4. Discussion

Previous studies have suggested that 1,25-(OH)2D3 plays a significant role in lung maturation before birth, as rat fetal lung extracts specifically bind 1,25-(OH)2D3 between days 18 and 22 of gestation, with a maximum in the few hours preceding birth followed by a decrease to barely detectable levels on the third day of life [1]. In addition, and based on cultures of rat fetal lung explants and of freshly isolated cells, type II pneumocytes have been found to express VDR and respond to low 1,25-(OH)2D3 doses by an increase in surfactant synthesis and secretion and by a decrease in glycogen content [2–4].

To get more evidence for a physiological function of 1,25-(OH)2D3 during lung maturation, we looked for the presence of VDR in freshly isolated lungs using cryosubstitution and immunogold electron microscopy. This was done on rat fetal lungs isolated 1 day before birth (day 21 of gestation), a time when type II pneumocytes at their different stages of maturation are simultaneously present. As expected, cells with type II pneumocyte characteristics recognized the monoclonal 9A7g anti-VDR antibody. Of interest, the immunogold labeling was restricted to type II cells, suggesting that they are specific target cells for 1,25-(OH)2D3 during fetal lung maturation. In agreement with these findings, no 1,25-(OH)2D3 binding capacity could be previously detected in fibroblast extracts isolated from rat fetal lung [2].

Subcellular localization of the immunogold particles strongly suggests the presence of VDR in the nucleus, but also in the cytoplasm and endoplasmic reticulum of type II cells. Because we used the 9A7g anti-VDR antibody, it is not possible to know whether this distribution reflects the intracellular trafficking of occupied or non-occupied VDR, but a similar distribution of the occupied VDR has

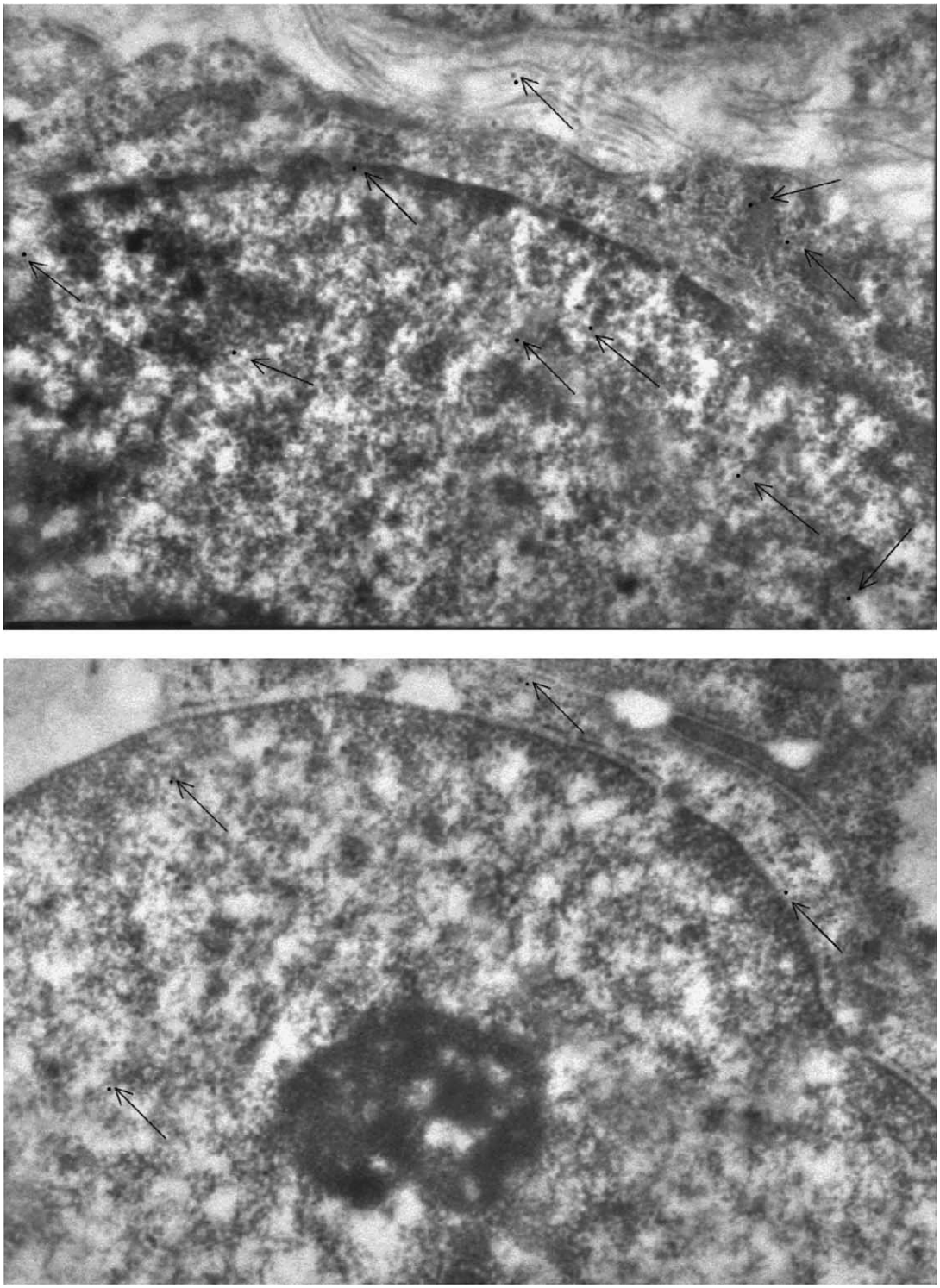

Fig. 1. Immunogold labeling on ultrathin cryosections of rat fetal lung using monoclonal antibody 9A7 gamma against VDR and a 15 nm protein G-gold complex. No immunogold labeling was observed in the absence of 9A7 gamma. The labeling was restricted to cells with the type II cells characteristics. As shown on the figure above, VDR labeling in type II cells was observed in the nucleus, the cytoplasm and the endoplasmic reticulum (arrows).

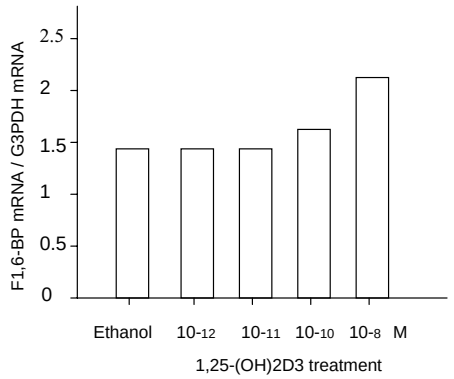

Fig. 2. Northern blot analysis of the fructose 1,6 bisphosphatase mRNA expression (1.7 Kb) in a human type II cell line (NCI-H441). These cells were treated with the indicated dose of 1,25-(OH)2D3 for 18 h in serum-free medium and then RNA was isolated. Northern blots were prepared and hybridized with labeled F 1,6BP and G3PDH cDNA probes.

been suggested using fluorescent calcitriol in human skin fibroblasts [12].

Previous work in fetal lung explants have shown a parallelism between VDR expression and onset of surfactant synthesis, suggesting a close association between VDR expression and type II cell maturation. The second aim of this ultrastructural study was to precise this relationship at the cellular level. We used cell glycogen content to evaluate type II cell maturation, as none of the fixation techniques tested before electronmicroscopy enabled simultaneous visualisation of osmiophilic lamellar bodies (OLBs), an indicator of surfactant production, and immunogold labeled VDR. Results of this cross-sectional analysis clearly show an association between VDR expression and individual stages of type II cell maturation. They suggest that 1,25-(OH)2D3 may be active at all stages of differentiation, but especially at the intermediate stage, when the physiological decrease in glycogen content begins.

The second aim of the present work was to test a possible effect of 1,25-(OH)2D3 on F1,6BP in lung. This enzyme has been recently cloned and identified in lung [5], and more specifically in human type II pneumocytes, but not in type I cells [6]. In addition, the F1,6BP gene bears a Vitamin D response element [7] and 1,25-(OH)2D3 induces gene expression in human differentiated monocytes [8]. The present findings of a F1,6BP mRNA expression in a human type II cell line (NCI-H441) bring more evidence for the involvement of F1,6BP in lung.

The results obtained with the NCl-H441 cell line also show their clear 1,25-(OH)2D3 binding capacity and suggest a positive 1,25-(OH)2D3 effect on F1,6BP expression. F1,6BP, a regulator of neoglucogenesis, increases glucose-6P production, which in turn may increase glycogen synthesis and/or neoglucogenesis. In the case this enzyme is expressed in type II pneumocytes during fetal life, an increase in glycogen synthesis via the 1,25-(OH)2D3-induced increase in F1,6BP expression appears unlikely. Indeed, VDR expression peaks at a time when the glycogen content of fetal type II cells decreases and 1,25-(OH)2D3 accelerates glycogenolysis [1,4]. The most likely hypothesis would therefore be that the increase in F1,6BP expression induced by 1,25-(OH)2D3 may favour synthesis of glucose-6P from noncarbohydrate precursors and thus facilitate the surfactant synthesis, as suggested in post-natal lung [6].

Taken together, the present results bring further evidence for a physiological role of 1,25-(OH)2D3 during type II pneumocyte maturation. Activation of F1,6BP may participate to the 1,25-(OH)2D3 action on surfactant synthesis via the gluconeogenesis pathway.

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