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Regulation of 25-hydroxyvitamin D_3 1α-hydroxylase, 1,25-dihydroxyvitamin D_3 24-hydroxylase and vitamin D receptor gene expression by 8-bromo cyclic AMP in cultured human syncytiotrophoblast cells☆

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Abstract

In vitro differentiation of human trophoblast cells is a dynamic process accompanied by increasing intracellular levels of cyclic AMP (cAMP). Signaling through cAMP in this tissue is central to hormone expression and cytodifferentiation. In the present study, we analyzed transcriptional regulation of key enzymes involved in vitamin D endocrine system during in vitro syncytiotrophoblast formation. Total RNA was isolated from human trophoblast cells and subjected to reverse transcription, polymerase chain reaction and Southern blot analysis using specific primers and radiolabeled probes. During syncytium formation 25-hydroxyvitamin D_3 1α-hydroxylase (CYP27B1) was decreased while vitamin D receptor (*VDR*) gene remained unaffected. No 1,25-dihydroxyvitamin D_3 24-hydroxylase (CYP24) transcription signal was detected. Nevertheless, incubations in the presence of 8-bromo cAMP (1.5 mM) resulted in CYP24 induction and CYP27B1 inhibition, respectively. The overall data showed that cultured human syncytiotrophoblasts express key enzymes involved in vitamin D metabolism, as well as VDR. The results support previous findings that human placenta is a source of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), which synthesis is regulated by common growth and developmental factors. The data also suggest a tissue-dependant differential regulation of *CYP27B1* gene expression by cAMP.

Keywords: Vitamin D; Placenta; Gene expression; Cell differentiation; Trophoblast

1. Introduction

During human placental development, cytotrophoblast cells differentiate into multinucleated syncytiotrophoblasts via cell fusion [1]. This pathway of differentiation is a key process in human pregnancy since most of the metabolic activities of the placenta, which are required for fetal growth and development, are associated with the acquisition of a syncytial phenotype [1]. Studies in trophoblast primary cultures have demonstrated that syncytial formation is cyclic AMP (cAMP) dependent [2]. In fact, intracellular levels of cAMP rise during in vitro citodifferentiation [3].

In addition, human syncytiotrophoblast cultures are able to synthesize the seco-steroid 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) [4–6]. This process is catalyzed by the placental 25-hydroxyvitamin D_3 1α-hydroxylase (1α-OHase or CYP27B1) [5], an enzyme that belongs to the mitochondrial cytochrome P450 family [7]. Furthermore, 1,25-dihydroxyvitamin D_3 24-hydroxylase (24-OHase or CYP24), a cytochrome involved in the main catabolic pathway of 1,25-$(OH)_2D_3$, and the vitamin D receptor (VDR) are also expressed in the human placenta [8,9]. Thus, the aim of this work was to study transcriptional regulation of three key components of the vitamin D endocrine system: 1α-OHase, 24-OHase and VDR, during in vitro differentiation of human trophoblasts cells. In addition, the effect of 8-bromo-cAMP (8-Br-cAMP), on *CYP27B1*, *CYP24* and *VDR* gene regulation was also studied.

2. Materials and methods

2.1. Trophoblast cell culture

The study protocol was approved by the Human Ethical Committee of the Institute. Term placentae (38–42 weeks

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of gestation) were obtained from normal pregnant women. The isolation and culture of trophoblasts was performed as described previously [5,10]. Briefly, villous tissue was digested with trypsin and DNase I, and cytotrophoblasts were separated on a Percoll gradient. Cells (2×10^6 cells/ml) were plated in Dulbecco's Modified Eagle Medium in humidified 5% CO_2–95% air at 37 °C. At different days of plating, cells were incubated in the presence or absence of 1.5 mM 8-Br-cAMP (Sigma Chemical Co., St. Louis, MO) during 24 h. Cell cultures were examined daily and both basal and 8-Br-cAMP stimulated human chorionic gonadotropin (hCG) secretion in culture media was measured by radioimmunoanalysis as previously described [4–6]. The area under the curve (AUC) of hCG secretion was calculated by the trapezoid method (SigmaStat; Jandel Scientific Software, Chicago, IL). Total protein content of cell cultures was determined by the method of Bradford using BSA as standard [11]. Statistical significance between comparisons was established using Student's *t*-test. A *P*-value equal or less to 0.05 was considered statistically significant.

2.2. PCR amplifications and Southern blot analysis

Total RNA was isolated from cultured trophoblast cells with Trizol® reagent (Invitrogen, USA) [12]. cDNA synthesis from 1 μg of total RNA was performed using reverse transcriptase (Invitrogen, USA), and then PCR amplifications were done with *Taq* DNA polymerase (Roche, Germany) and the following sense and antisense primers: β*hCG* (5′-CGCACCAAGGATGGAGA-3′ and 5′-GCCTTTATTGTGGAGGA-3′); *CYP27B1* (5′-GTTGCTATTGGCGGGAGTGGAC-3′ and 5′-GTGACACAGAGTGACCAGCGTAT-3′); *CYP24* (5′-CCTCGTGTTG TATGAGAAGAGATT-3′ and 5′-TTTAAATACGGCATATTCCTCAAA-3′); and *VDR* (5′-TCCTCCTGCTCAGATCACTGT-3′ and 5′-CTCCTCCTCATGCAAGTTCAG-3′); which yielded a 494, 298, 489 and 438 bp RT-PCR products, respectively. Normalization was performed by the amplification of cyclophilin mRNA with the following sense and antisense primers: 5′-CCCCACCGTGTTCTTCGACAT-3′ and 5′-AGGTCCTTACCGTTCTGGTCG-3′, which yielded a 453 bp RT-PCR product. The RT-PCR products were separated in agarose gels, blotted onto nylon membranes and hybridized with probes radiolabeled with [^{32}P]-dCTP. Specific probes were obtained by RT-PCR from human placental tissue using the following upper and lower primers: for the 494 bp βhCG probe (5′-CGCACCAAGGATGGAGA-3′ and 5′-GCCTTTATTGTGGAGGA-3′); for the 183 bp CYP27B1 probe (5′-TTGGGGATAATATAGTCACCCAC-3′ and 5′-CCACTCAGAGATCACAGCTGC-3′); for the 170 bp CYP24 probe (5′-TCAGCAGCCTAGTGCAGATT-3′ and 5′-TTTGTTGCACTTGGGGATTA-3′); for the 172 bp VDR probe (5′-ACAGCATCCAAAAGGTCATTG-3′ and 5′-TAGTCTTGGTTGCCACAGGTC-3′); and for the 187 bp cyclophilin probe (5′-CACACGCCATAATGGCACTGGTGG-3′ and 5′-AAAGACCACATGCTTGCCATCCAGC-3′), respectively. In all cases, after 18 h hybridization, filters were washed and exposed to X-ray films.

3. Results

Microscopic examination of cell cultures showed that within 3 days after plating the cultured cytotrophoblasts formed cell aggregates conformed mostly of larger areas containing multiple nuclei with very little, if any, single mononuclear cells. By day 3, multinucleated cells seemed to form a network that corresponded to functional syncytiotrophoblasts, as determined by their ability to secrete hCG. Fig. 1 shows the area under the curve of hCG released during 96 h of culture. As depicted, cell cultures were able to synthesize basal amounts of hCG and the addition of 8-Br-cAMP significantly increased the content of hCG in the culture media. Fig. 2 shows the temporal pattern of transcription of the β subunit of hCG (βhCG) during trophoblast differentiation in the presence or absence of 8-Br-cAMP. A specific 494 bp βhCG cDNA fragment was obtained in basal conditions with a pattern according to the released hormone (Fig. 2A). The relative abundance of βhCG mRNA (Fig. 2C) was obtained by normalizing the specific βhCG band intensity with the 453 bp signal generated for the housekeeping gene cyclophilin (Fig. 2B). As shown, 8-Br-cAMP increased βhCG mRNA, compared with cultures in the absence of the cyclic nucleotide analogue (Fig. 2C).

In order to study the regulation of key elements of the vitamin D endocrine system during trophoblast differentiation, total RNA was extracted from cultured cells at different days from plating and subjected to RT-PCR and Southern blot analysis. Fig. 3 shows the temporal pattern of expression of *1α-OHase* and *24-OHase* genes. As depicted, the 298 bp product of *CYP27B1* gene was decreased during the process of trophoblasts differentiation under basal conditions (Fig. 3A), and the addition of 8-Br-cAMP further inhibited transcription of this gene (Fig. 3D). In contrast, *CYP24* gene was up regulated by 8-Br-cAMP addition

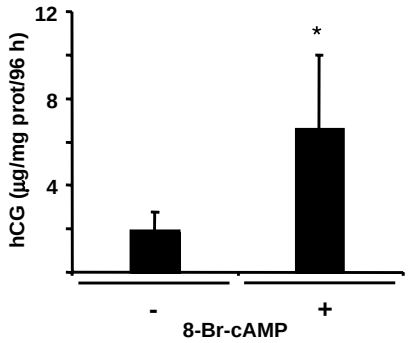

Fig. 1. The hCG secretion by trophoblast cells in culture. Area under the curve of hCG released during 96 h in the absence (−) or presence (+) of 8-Br-cAMP. (*) $P < 0.05$ vs. control.

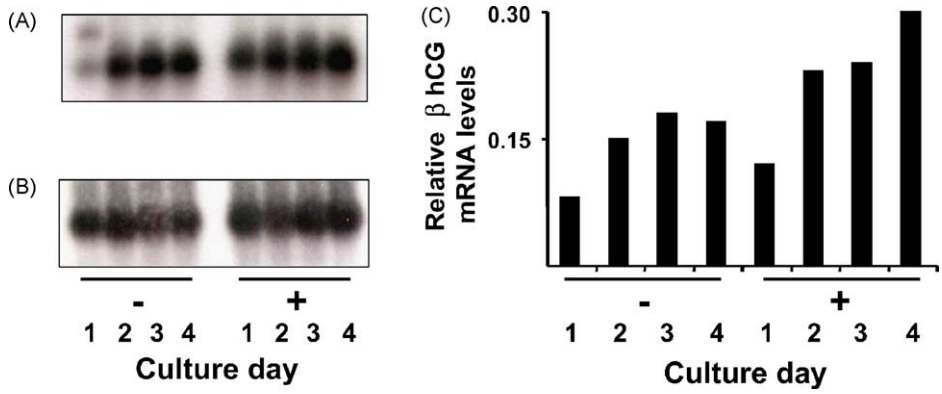

Fig. 2. Time course of expression of βhCG mRNA in cultured trophoblast cells. Total RNA was obtained daily from cultured cells during different days in the absence (−) or presence (+) of 8-Br-cAMP and then subjected to RT-PCR as described in Section 2. (A) Southern blot analysis of 494 bp βhCG RT-PCR products. (B) RT-PCR amplifications of cyclophilin. (C) Normalization of relative optical densities of RT-PCR products of βhCG and cyclophilin.

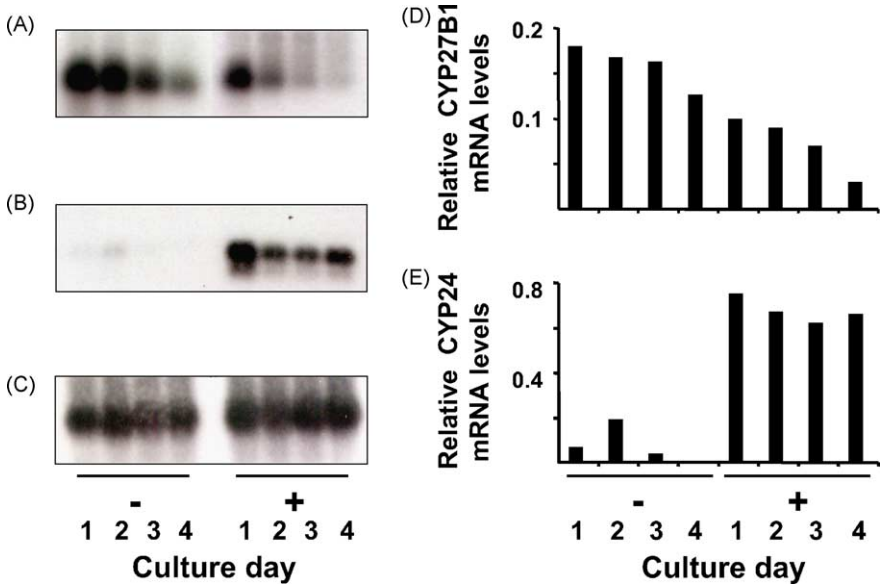

Fig. 3. Expression of *CYP27B1* and *CYP24* genes in the presence (+) or absence (−) of 8-Br-cAMP in cultured trophoblast cells. Total RNA was obtained at different days of culture and subjected to RT-PCR analysis using specific cDNA probes for *CYP27B1* (A), *CYP24* (B) and cyclophilin (C), respectively. Normalization of the relative optical densities of the 298 bp *CYP27B1* RT-PCR product with the 453 bp cyclophilin fragment is shown in (D). Normalization of 489 bp *CYP24* RT-PCR product with cyclophilin signal is shown in (E).

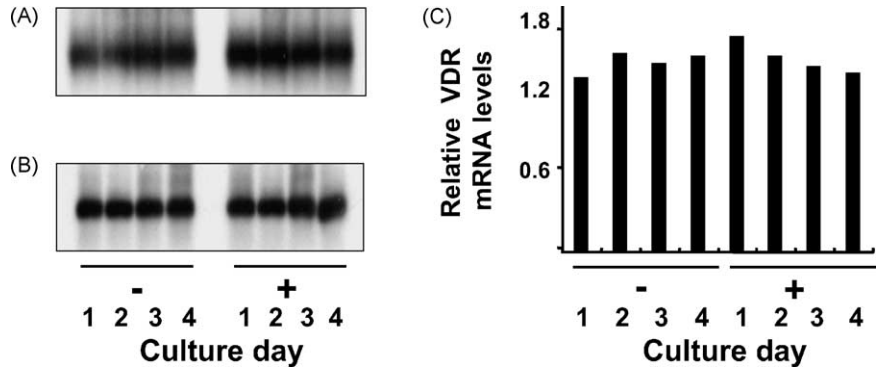

Fig. 4. Expression of VDR mRNA in cultured trophoblast cells. Daily total RNA was obtained from cultured cells in the absence (−) or the presence (+) of 8-Br-cAMP and then subjected to RT-PCR as described in Section 2. This yielded VDR-products of 438 bp (A) and cyclophilin 453 bp products (B), respectively. The Southern blot was then probed with specific nested cDNA fragments. Normalization of relative optical densities of RT-PCR products is shown in Panel C.

(Fig. 3B and E). As can be seen in Fig. 3B, the 489 bp cDNA fragment of 24-OHase mRNA was barely detectable under basal conditions but clearly visible under 8-Br-cAMP stimuli. On the other hand, VDR mRNA remained without changes throughout the differentiation process, and the addition of 8-Br-cAMP slightly increased VDR mRNA levels at 24 h of culture (Fig. 4A). Normalization was carried out with cyclophilin (Fig. 4B and C).

4. Discussion

Although calcium homeostasis is a main issue for the maternal organism during pregnancy, very few studies have been aimed at investigating placental vitamin D metabolism. The placenta is active in calcium transport, and expresses *VDR*, *CYP24* and *CYP27B1* [5,9,13]. In the present study, we provide evidence for the role of cAMP on transcriptional regulation of three key enzymes involved in vitamin D metabolism in human placenta.

Under the experimental conditions used in this study, β*hCG* expression and hCG secretion were analyzed in all culture days. We observed that hCG concentrations progressively increased with the acquisition of a syncytial phenotype, a process that was further enhanced in the presence of 8-Br-cAMP (Fig. 2).

The presence of cAMP responsive elements (CREs) have been described in the promoter regions of *CYP24* and *CYP27B1* genes [14,15], which implies that cAMP-dependant regulation is feasible. In fact, in the kidney, cAMP and forskolin stimulate *CYP27B1* and inhibit *CYP24* gene expression [16,17]. On the contrary, in the experimental model used herein, incubation of syncytiotrophoblasts in the presence of 8-Br-cAMP resulted in *CYP27B1* inhibition and *CYP24* stimulation, which suggest a tissue-specific regulation of these genes. In addition, a progressive and spontaneous decline of *CYP27B1* expression was observed during trophoblast differentiation under basal conditions. This finding is in line with previous studies showing that intracellular content of cAMP rises throughout in vitro syncytial formation [18]. Indeed, activation of cAMP dependant kinases is known to be crucial for the trophoblast differentiation [2].

Interestingly, the observation that *CYP24* transcriptional activity could not be detected under basal conditions in none of the different days of culture (Fig. 3B), suggests that generation of endogenous cAMP during cytotrophoblasts differentiation was not sufficient enough to induce *CYP24* expression. Nevertheless, the fact that 8-Br-cAMP significantly switched on *CYP24* gene expression, also suggests that this gene is regulated via the PKA signaling pathway, independently of 1,25-$(OH)_2D_3$. Indeed, it is known that the major route of 24-hydroxylase induction involves the combined action of 1,25-$(OH)_2D_3$ and *VDR* [19,20]. Consequently, cellular expression of *CYP24* is tightly linked to the coexpression of *VDR*. Most cells that contain VDR express basal levels of *CYP24*; however, in this study *CYP24* transcription was not detected in the absence of 8-Br-cAMP, even when *VDR* gene expression was observed (Figs. 3B and 4 respectively). This finding may indicate the role of cAMP on VDR actions upon *CYP24* gene expression. In addition, the presence of 8-Br-cAMP in the first day of culture induced a slight increase on *VDR* gene transcription, which was in agreement with the presence of CREs in the promoter region of this gene [21,22].

Finally, it has been established that 1,25-$(OH)_2D_3$ is involved in the regulation of placental lactogen expression, decidualization of the endometrium, and calcium transport in the placenta [23–25]. Furthermore, since 1,25-$(OH)_2D_3$ is considered as an immunosuppressor agent and acts as a regulator of the synthesis of several cytokines [26], it may contribute to the establishment and maintenance of the fetoplacental unit. The overall data point out the importance of future studies on the physiological meaning of vitamin D metabolism during the process of placental citodifferentiation.

In summary, the present study demonstrates a tissue-specific regulation of key cytochromes involved in the vitamin D endocrine system and highlights the role of cAMP on *CYP27B1* and *CYP24* gene regulation in the placenta.

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