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Vitamin D and growth hormone regulate growth hormone/insulin-like growth factor (GH–IGF) axis gene expression in human fetal epiphyseal chondrocytes

M. Fernández-Cancio [a,*,1], L. Audi [a,1], A. Carrascosa [a], N. Toran [b], P. Andaluz [a], C. Esteban [a], M.L. Granada [c]

[a] Pediatric Endocrinology Research Unit, Research Institute, Hospital Vall d'Hebron, Autonomous University of Barcelona, CIBERER (Centro de Investigación en Red de Enfermedades Raras), Instituto de Salud Carlos III, Barcelona, Spain
[b] Dept. of Pathology, Hospital Vall d'Hebron, CIBERER, Barcelona, Spain
[c] Hormonal Laboratory, Hospital Germans Trias-Pujol, Badalona, Spain

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ABSTRACT

M.L. Gordon

Objective: Cell proliferation and gene expression regulation were studied in human fetal epiphyseal chondrocytes to ascertain the involvement of GH–IGF axis components in human fetal growth regulation by 1,25-dihydroxyvitamin D_3 (VitD) and growth hormone (GH).

Design: Chondrocytes from primary cultures were plated in serum-free medium for 48 h and incubated for a further 48 h with VitD (10^{-11} to 10^{-6} M) and/or IGF-I (100 ng/ml) and/or GH (500 ng/ml). We analyzed ^{3}H-thymidine incorporation into DNA and IGF-I, IGFBP-3, GHR, SOX9, COL2A1, aggrecan and COMP gene expression by real-time quantitative PCR.

Results: VitD dose-dependently and significantly inhibited ^{3}H-thymidine incorporation whereas GH had no effect on proliferation and, when combined with VitD, the same inhibition was observed as with VitD alone. IGF-I (100 ng/ml) significantly stimulated proliferation and opposed inhibition by VitD. VitD dose-dependently stimulated IGF-I (11.1 ± 19.8 at VitD 10^{-6} M), IGFBP-3 (2.6 ± 0.9), GHR (3.8 ± 2.8) and COMP (1.5 ± 0.6) expression whereas it inhibited SOX9 (0.7 ± 0.2), COL2A1 (0.6 ± 0.3) and aggrecan (0.6 ± 0.2) expression and had no significant effect on IGF-II. IGF-I stimulated IGF-I, IGFBP-3, SOX9, COL2A1 and aggrecan expression and opposed COL2A1 and aggrecan gene expression inhibition by VitD. GH alone had no effect on gene expression whereas, in the presence of VitD, significantly-increased IGF-I expression stimulation was observed above values obtained with VitD alone (17.5 ± 7.4).

Conclusions: Our results suggest that VitD regulation of fetal growth cartilage could have consisted of parallel enhancing of cell differentiation and conditioning to a phenotype more sensitive to regulation by other hormones such as GH as shown by increased GHR and IGF-I expression, but not by IGF-II expression which was not regulated.

1. Introduction

Chronic vitamin D deficiency, whether due to nutritional deprivation or lack of exposure to sunlight, leads to typical bone diseases such as rickets in the young or osteomalacia in the adult, and the genetic disorder related to mutations in the vitamin D receptor (VDR) gene results in the appearance of 1,25-dihydroxyvitamin D_3 (VitD)-resistant rickets type II [1]. Collectively, these observations imply that VitD plays a major role in bone biology. This role is believed to consist principally of maintaining systemic and local calcium concentrations since rachitic lesions can be cured by calcium administration in VitD-resistant patients [2]. However, recent molecular genetic studies have revealed a direct role for VitD on growth plate chondrocytes [3].

Other actions of VitD, demonstrated to be of biological concern, include regulation of growth and differentiation in various tissues and cells [4–6], and regulation of the immune system. VitD inhibits cell proliferation and induces a more differentiated phenotype in most cells and recent evidence revealed that VitD exhibits antiproliferative and differentiation-inducing effects and these findings suggest a potential use for VitD in the treatment of cancer [7].

Rickets patients are often growth-retarded; however, very few reports described the effects of VitD supplementation and rickets cure on skeletal growth and growth-related hormone secretion patterns [8]. Several hormones, of which GH is generally accepted as the most important, are crucial for normal postnatal

* Corresponding author. Address: Unidad Investigación Endocrinología Pediátrica, Institut de Recerca, Hospital Vall d'Hebron, Paseo Vall d'Hebron 119, Barcelona 08035, Spain. Tel./fax: +34 93 489 40 30.
E-mail address: mfernand@ir.vhebron.net (M. Fernández-Cancio).
[1] Both authors contributed equally to this work.

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longitudinal bone growth [9]. A question widely discussed over the last two decades has been whether GH acts on tissues directly or whether its effect is mediated by systemic and/or local IGF-I [10].

Locally-produced IGF axis components, including IGF-I, IGF-II, their binding proteins (IGFBPs)-1 to -6 and their receptors, play a key role in longitudinal bone growth, as shown by *in vitro* and *in vivo* experiments [9,11,12]. Disruption of IGF-I or IGF-II signaling results in prenatal growth retardation [13]. Several *in vitro* data have shown expression of both IGFs in the growth plate [12,14,15], while numerous *in vivo* studies have yielded conflicting results, with IGF-II being the most abundant IGF, whereas IGF-I could only be detected depending on animal ages or techniques used [16–20]. We recently demonstrated that human fetal epiphyseal chondrocytes expressed GH–IGF axis components, including IGF-I, IGF-II, IGFBP-3, GHR and IGF–IR, and that *in vitro* these genes, except IGF-II, were regulated by glucocorticoids and IGF-I [21].

To elucidate the direct effects of VitD on human fetal epiphyseal cartilage cells, we analyzed its dose-dependent effects on proliferation and expression of the GH–IGF axis and chondrocyte phenotype genes. In addition, interaction between VitD and GH or IGF-I effects was also analyzed to demonstrate that, under the experimental conditions used, VitD was required for IGF-I gene expression stimulation by GH.

2. Methods

2.1. Material/reagents

Ham F-12 medium and fetal calf serums (FCS) were purchased from GIBCO. Plastic plates and flasks were obtained from NUNC. Calcitriol (1,25-dihydroxyvitamin D_3) was a generous gift from Solvay Duphar and recombinant IGF-I and GH a generous gift from Pharmacia. 6-^{3}H-thymidine was obtained from Amersham Biosciences.

2.2. Cell culture

Tibial and femoral epiphyseal cartilage from human fetuses (14–22 weeks old; 13 females and 12 males), products of legal terminations, were collected with informed parental consent within 12 h postmortem. The protocol was approved by the Hospital Ethics Committee. Only fetuses with normal karyotype and diagnoses which do not affect skeletal growth were retained. The epiphyseal cartilage was cleaned of surrounding tissue under sterile conditions. The remaining cartilage was cut into thin slices and finely chopped with a surgical blade. Chondrocytes were released by enzymatic digestion and cultured as previously described [21,22]. Briefly, a chondrocyte suspension was obtained after enzymatic digestion with trypsin and collagenase. Approximately 4000–7000 cells/cm^2 were plated in a 75 cm^2 plastic culture flask with 12 ml of Ham F-12 medium. The culture media were supplemented with 10% FCS and 25 IU/ml of penicillin and streptomycin. The flasks were maintained at 37 °C in an atmosphere of 5% CO_2 in humid air. The cultures were fed by changing the medium every three days.

2.3. ^{3}H-thymidine incorporation into DNA

Studies were carried out as previously described [21–23]. Primary confluent chondrocytes were trypsinized and plated in 96-microwell tissue culture plates at a density of 60,000 cells/cm^2 in 0.3 ml Ham F-12 serum- and antibiotic-free medium. After 48 h, medium was aspirated, and chondrocytes were incubated for 48 h with VitD at increasing concentrations (10^{-11} to 10^{-6} M) and/or IGF-I (100 ng/ml) and/or GH (500 ng/ml). Control wells were incubated with medium containing the same amount of ethanol (0.4%) and BSA (0.1%) as VitD, IGF-I and GH conditions. ^{3}H-thymidine (5 μCi/ml) was added for the last 24 h of incubation. Four different wells were prepared for each condition and results were expressed as percent of mean radioactivity in four wells with respect to the control condition (100%). Twenty-five experiments were performed in chondrocyte cultures from different fetuses (13 females and 12 males; GA: 20.7 ± 2.1 w).

2.4. Gene expression

For gene expression study, primary confluent chondrocytes were trypsinized and 42,000 cells/cm^2 were plated in 6-well tissue culture plates with 2 ml Ham F-12 serum- and antibiotic-free medium. After 48 h, this medium was aspirated and chondrocytes were incubated for 48 h with VitD (10^{-11} to 10^{-6} M) and/or IGF-I (100 ng/ml) and/or GH (500 ng/ml). A time-dependent study was conducted (1, 2, 4, 24 and 48 h) for VitD 10^{-6} M and VitD 10^{-6} M with GH (500 ng/ml) effects. Control wells were incubated with medium containing the same amount of ethanol (0.4%) and BSA (0.1%) as VitD, IGF-I and GH conditions. Cells were trypsinized and 350 μl of guanidine isothiocyanate solution were added to the pellet.

Total RNA extraction and real-time quantitative PCR were carried out as previously described [21]. Assays-on-Demand used were Hs00153126_m1 (IGF-I), Hs00171254_m1 (IGF-II), Hs00426287_m1 (IGFBP-3), Hs00174872_m1 (growth hormone receptor, GHR), Hs00165814_m1 (SOX9), Hs00264051_m1 (collagen type 2 1A, COL2A1), Hs00153936_m1 (aggrecan), and Hs00164359_m1 (cartilage oligomeric matrix protein, COMP) (http://products.appliedbiosystems.com). Real-time fluorescence was monitored using ABI PRISM 7000 instrument. Relative expression levels of the various transcripts were generated for each one and relative abundance was then determined by comparing the cycle threshold (C_t) values for each reaction with comparative C_t method ($2^{\Delta\Delta Ct}$) with respect to the control condition. Variations in input cDNA mass were corrected by normalizing all data with the corresponding huCYC levels. All measurements were performed in triplicate, in eight independent experiments from eight different fetuses (5 females and 3 males) for incubations at 48 h and in four independent experiments (4 males) for time-dependent VitD 10^{-6} M and VitD 10^{-6} M with GH (500 ng/ml) effects.

2.5. IGF-I and IGFBP-3 protein assays

IGF-I and IGFBP-3 protein concentrations were assayed in culture media from one experiment at the end of the 48 h incubation protocol. IGF-I was measured with an IGF-I ELISA Kit from Diagnostic Systems Laboratories, Inc. with previous IGFBP interference separation and IGFBP-3 was measured with NICHOLS Institute Diagnostics RIA kit for human IGFBP-3.

2.6. Statistical analysis

Results were expressed as mean ± SD or as box plot (10th, 25th, 50th, 75th, 90th percentiles). Differences between controls and each experimental condition were analyzed with the non-parametric Paired Sign Test with StatView 4.5 program (Abacus Concepts, CA, USA).

3. Results

3.1. ^{3}H-thymidine incorporation into DNA (Fig. 1)

VitD dose-dependently and significantly inhibited proliferation from 10^{-10} M to 10^{-6} M (41.9 ± 13.1%, $p < 0.0001$ at 10^{-6} M)

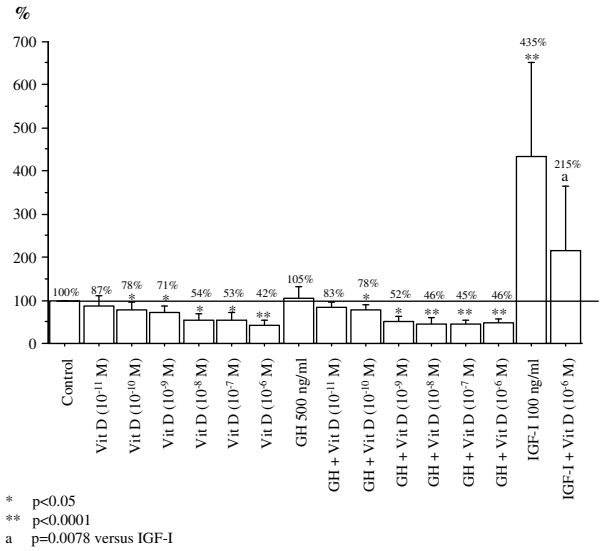

Fig. 1. ^{3}H-thymidine incorporation into DNA. Bar chart results (mean + SD) of ^{3}H-thymidine incorporation into DNA obtained under different experimental conditions. Chondrocytes from human fetal epiphyseal growth plate were plated at a density of 60,000 cells/cm^2 with Ham F-12 serum-free medium for 48 h. Cells were incubated for 48 h with VitD (10^{-11} to 10^{-6} M) and/or IGF-I (100 ng/ml) and/or GH (500 ng/ml). Control wells were incubated with medium containing the same amount of ethanol and BSA as VitD, IGF-I and GH conditions. Four different wells were prepared for each condition. ^{3}H-thymidine was added for the last 24 h of incubation. Results for each condition were expressed as percent of mean radioactivity in four wells with respect to control condition (100%). Twenty-five experiments were performed in chondrocyte cultures from different fetuses (13 females and 12 males; GA: 20.7 ± 2.1 w). ($^*p < 0.05$, $^{**}p < 0.0001$, $^ap = 0.0078$ vs. IGF-I condition; VitD: vitamin D, GA: gestational age).

whereas GH (500 ng/ml) had no effect. Combined incubation with VitD and GH significantly inhibited proliferation, as did VitD alone. IGF-I (100 ng/ml) significantly stimulated DNA synthesis (434.9 ± 217.6%, $p < 0.0001$) and partially opposed proliferation inhibition by VitD 10^{-6} M (215.4 ± 149.9%).

3.2. Gene expression

3.2.1. IGFs, IGF-II and IGFBP-3

VitD significantly stimulated IGF-I and IGFBP-3 gene expression. VitD stimulation of IGF-I expression was observed to be dose-dependent from 10^{-10} to 10^{-6} M, although it was statistically significant from 10^{-8} M owing to its wide range of variation (from 1.5 to 56.0 at 10^{-6} M) (11.1 ± 19.8 at VitD 10^{-6} M) (Fig. 2A). VitD stimulation of IGFBP-3 expression was also dose-dependent and statistically significant from 10^{-11} to 10^{-6} M (2.6 ± 0.9, $p < 0.0005$ at 10^{-6} M) (Fig. 2B). Mean increase in IGF-I mRNA was 4.4-fold higher than that of IGFBP-3 mRNA at VitD 10^{-6} M. VitD had no significant effect on IGF-II expression (Fig. 2C).

GH had no significant effect on IGF-I, IGF-II or IGFBP-3 gene expression. However, the combination of GH and VitD significantly-increased IGF-I gene expression above the stimulation produced by VitD alone (17.5 ± 7.4 at VitD 10^{-6} M + GH 500 ng/ML) ($p < 0.05$, above each VitD condition alone from 10^{-9} M to 10^{-6} M) (Fig. 2A). This combination had a similar effect on IGFBP-3 gene expression as did VitD alone. Again, as for VitD or GH alone, the combination had no significant effect on IGF-II gene expression (Fig. 2C).

Time-dependent experiments showed that VitD 10^{-6} M had significantly stimulated IGF-I expression at 48 h (2.27 ± 0.69), whereas IGFBP-3 stimulation was detected from 24 h onwards (4.19 ± 1.23). Combination of VitD 10^{-6} M with GH (500 ng/ml) produced a significant stimulation of IGF-I expression from 4 h (3.23 ± 1.4).

IGF-I (100 ng/ml) stimulated IGFBP-3 gene expression and combined incubation of IGF-I with VitD 10^{-6} M had an additive effect on IGF-I and IGFBP-3 gene expression (Fig. 2A and B). This combination had no significant effect on IGF-II gene expression, and neither did VitD or IGF-I alone (Fig. 2C).

3.2.2. GHR

VitD strongly stimulated GHR expression which was significant from 10^{-10} M (3.8 ± 2.8, $p < 0.005$ at 10^{-6} M). GH (500 ng/ml) or IGF-I (100 ng/ml) had no effect on GHR expression. Combined incubation with VitD and GH (500 ng/ml) or IGF-I (100 ng/ml) stimulated GHR expression, as did VitD alone (Fig. 2D).

3.2.3. SOX 9 and matrix proteins (Table 1)

VitD 10^{-11} M had a variable effect on SOX9, COL2A1 and aggrecan expression but higher concentrations significantly inhibited expression of these genes (0.7 ± 0.21, $p < 0.05$; 0.56 ± 0.32, $p < 0.05$; 0.59 ± 0.23, $p < 0.05$, respectively, at 10^{-6} M), while they significantly stimulated COMP expression (1.53 ± 0.62, $p < 0.05$ at 10^{-6} M). GH alone had no significant effect. IGF-I (100 ng/ml) stimulated SOX9, COL2A1 and aggrecan expression (1.83 ± 0.77; 3.08 ± 1.31; 3.40 ± 1.94, respectively). Neither IGF-I nor GH could oppose VitD inhibition of SOX9 expression (0.33 ± 0.15; 0.68 ± 0.30, $p < 0.05$ at 10^{-6} M), whereas IGF-I did impede the inhibition produced by VitD on COL2A1 and aggrecan expression (2.14 ± 0.66; 1.28 ± 0.63, respectively).

3.3. IGF-I and IGFBP-3 protein levels

IGF-I protein released in the culture medium over the 48 h incubation period was increased under VitD 10^{-6} M (8.0 ng/ml) over the control condition (4.1 ng/ml). The corresponding mRNA as detected at the end of the 48 h was also increased (3.38 with control being 1).

IGFBP-3 protein released was increased at VitD 10^{-6} M (191 ng/ml) over the control condition (139 ng/ml). The corresponding mRNA was also increased (2.52) at the end of incubation.

4. Discussion

In the present study, VitD was found to inhibit DNA synthesis in a dose-dependent manner as shown by ^{3}H-thymidine incorporation from 10^{-11} to 10^{-6} M, and this inhibitory effect has also been shown by other authors in different cell types including growth plate chondrocytes [24] although Krohn et al. [25] showed that VitD at a lower concentration (10^{-12} M) stimulated DNA synthesis of epiphyseal chondrocytes from young male rats. This inhibitory action of VitD at 10^{-6} M was completely opposed by IGF-I (100 ng/ml) although VitD diminished the stimulatory effect of IGF-I. The DNA synthesis stimulatory effect of IGF-I has been demonstrated in normal cultured cells, including rat [26,27] and human [21,28,29] growth plate cells and the opposing effect of IGF-I on DNA synthesis inhibition by VitD was previously shown to need a high IGF-I concentration (50 ng/ml) [30]. The mechanism for VitD DNA synthesis regulation may be mediated through targeting of several key regulators governing the G_1/S transition, such as p21, and/or an increase in apoptosis. The p21 induction by VitD may be direct or secondary to IGFBP-3 induction. Whether the observed inhibition of cell proliferation by VitD in our culture system was mediated directly or indirectly by increased IGFBP-3 secretion was not ascertained. However, although IGF-I expression stimulation was on average four times higher than that of IGFBP-3, IGFBP-3 protein level reached in the culture media under VitD stimulation was in the order of 0.4×10^{-8} M (191 ng/ml) and at this concentration exogenous IGFBP-3 has been shown to inhibit ^{3}H-thymidine

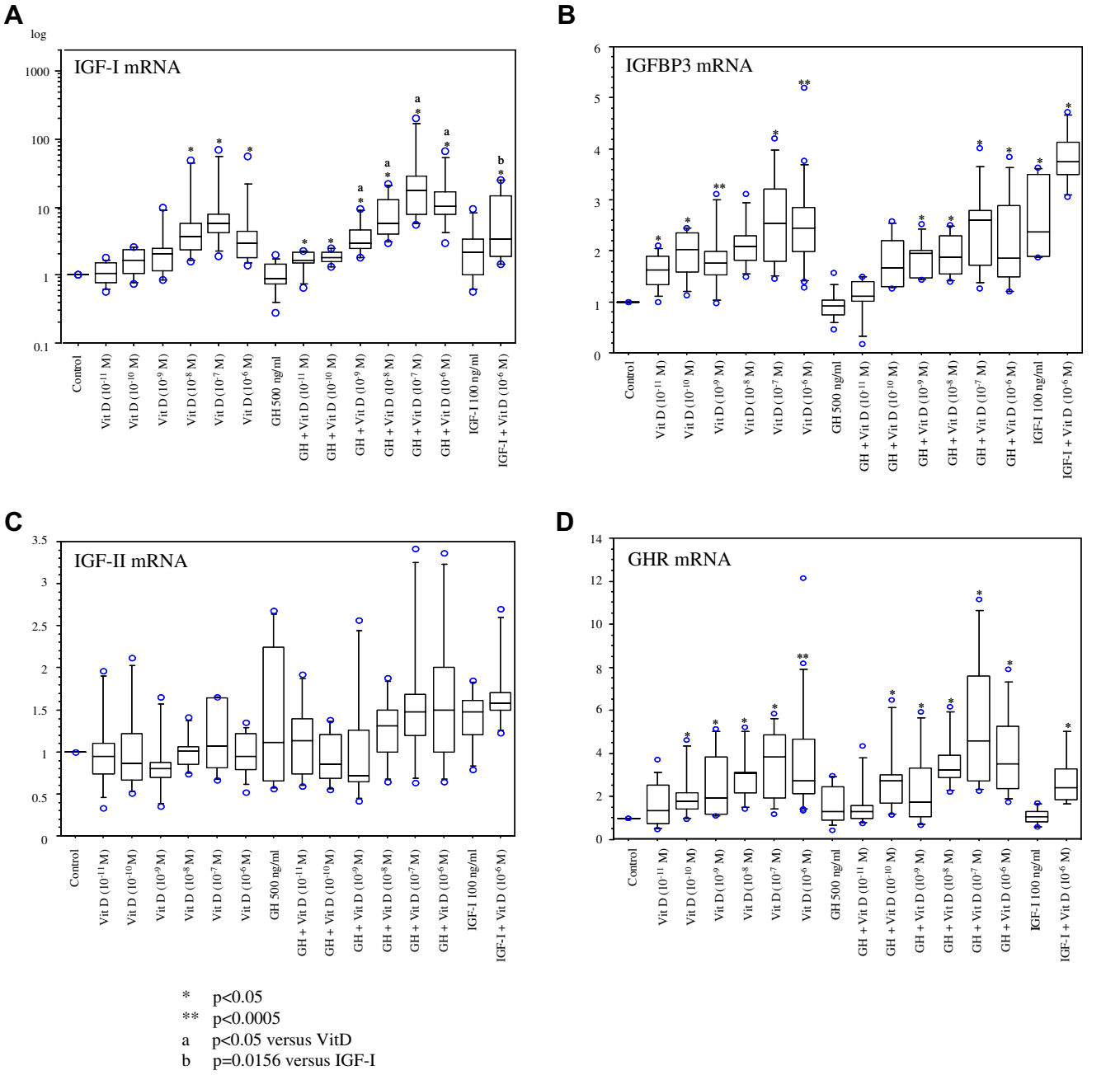

Fig. 2. IGF-I, IGFBP-3, IGF-II and GHR gene expression regulation. Box plot (10th, 25th, 50th, 75th, 90th percentiles) of IGF-I, IGFBP3, IGF-II and GHR gene expression regulation results obtained under different experimental conditions. IGF-I mRNA was expressed as log scale. Chondrocytes from human fetal epiphyseal growth plate were plated at a density of 42,000 cells/cm^2 with Ham F-12 serum-free medium for 48 h. Cells were incubated for 48 h with VitD (10^{-11} to 10^{-6} M) and/or IGF-I (100 ng/ml) and/or GH (500 ng/ml). Control wells were incubated with medium containing the same amount of ethanol and BSA as VitD, IGF-I and GH conditions. Cells were trypsinized, total RNA was extracted and reverse-transcripted. cDNA for each gene was quantified by real-time quantitative PCR. Relative expression levels of the various transcripts were generated for each one and relative abundance was then determined by comparing the cycle threshold (C_t) values for each reaction with comparative C_t method ($2^{\Delta\Delta Ct}$) with respect to the control condition. Variations in input cDNA mass were corrected by normalizing all data with the corresponding huCYC levels. All measurements were performed in triplicate and in eight independent experiments from eight different fetuses (5 females and 3 males) ($^{*}p < 0.05$, $^{**}p < 0.0005$, $^{a}p < 0.05$ vs. VitD condition, $^{b}p = 0.0156$ vs. IGF-I condition; VitD: vitamin D).

incorporation into DNA (data not shown). Moreover, a high IGF-I concentration (100 ng/ml) was able to overcome VitD cell proliferation inhibition (our results) whereas a low concentration of 1 ng/ml [30] and 8 ng/ml (0.1×10^{-8} M) of IGF-I vs. 191 ng/ml (0.4×10^{-8} M) for IGFBP-3 (our VitD 10^{-6} M condition) could not.

In our culture system, GH (500 ng/ml) had no effect on proliferation or modified the dose-dependent VitD inhibition, similarly to results from Hutchison et al. [31] who failed to demonstrate a direct effect of GH on bovine postnatal growth plate chondrocytes, and in contrast to Ohlsson et al. [32] who showed that GH stimulated cell proliferation in chondrocytes from fetal rat tibia.

In this study VitD was found to stimulate IGF-I, IGFBP-3, GHR and COMP gene expression, whereas it inhibited SOX9, COL2A1 and aggrecan and had no effect on IGF-II expression. In addition, it had no effect on CBFA1 (core-binding factor 1), COL10A1, SHOX (short stature homeobox-containing) or STAT5b (signal transducer

Table 1
Relative expression levels of SOX9, COL2A1, aggrecan and COMP transcripts expressed as mean ± SD under different experimental conditions.

Experimental conditions	SOX9	COL2A1	Aggrecan	COMP
Control	1.00	1.00	1.00	1.00
VitD 10^{-11} M	1.16 ± 0.62	1.06 ± 0.51	0.81 ± 0.28*	0.92 ± 0.42
VitD 10^{-10} M	0.84 ± 0.35	0.85 ± 0.15*	0.82 ± 0.17*	1.03 ± 0.45
VitD 10^{-9} M	0.66 ± 0.18*	0.58 ± 0.30*	0.64 ± 0.26*	0.87 ± 0.53
VitD 10^{-8} M	0.76 ± 0.26*	0.63 ± 0.26*	0.63 ± 0.17*	1.66 ± 0.77*
VitD 10^{-7} M	0.83 ± 0.32*	0.51 ± 0.09*	0.57 ± 0.17*	1.55 ± 0.87*
VitD 10^{-6} M	0.70 ± 0.21*	0.56 ± 0.32*	0.59 ± 0.23*	1.53 ± 0.62*
GH 500 ng/ml	0.82 ± 0.47	0.88 ± 0.26	0.85 ± 0.34	1.26 ± 0.60
GH + VitD 10^{-11} M	0.63 ± 0.24*	0.77 ± 0.17*	0.72 ± 0.25*	1.24 ± 0.81
GH + VitD 10^{-10} M	0.70 ± 0.08*	0.82 ± 0.18*	0.69 ± 0.20*	1.43 ± 0.49*
GH + VitD 10^{-9} M	0.74 ± 0.21*	0.59 ± 0.24*	0.61 ± 0.26*	1.01 ± 0.35
GH + VitD 10^{-8} M	0.66 ± 0.19*	0.60 ± 0.33*	0.54 ± 0.19*	1.15 ± 0.54
GH + VitD 10^{-7} M	0.63 ± 0.25*	0.50 ± 0.20*	0.46 ± 0.07*	1.57 ± 0.34*
GH + VitD 10^{-6} M	0.68 ± 0.30*	0.45 ± 0.11*	0.48 ± 0.07*	1.24 ± 0.47
IGF-I 100 ng/ml	1.83 ± 0.77	3.08 ± 1.31	3.40 ± 1.94	0.70 ± 0.49
IGF-I + VitD10^{-6} M	0.33 ± 0.15*	2.14 ± 0.66	1.28 ± 0.63	1.44 ± 0.45*

Chondrocytes from human fetal epiphyseal growth plate were plated at a density of 42,000 cells/cm^2 with Ham F-12 serum-free medium for 48 h. Chondrocytes were incubated for 48 h with VitD (10^{-11} to 10^{-6} M) and/or IGF-I (100 ng/ml) and/or GH (500 ng/ml). Control wells were incubated with medium containing the same amount of ethanol and BSA as VitD, IGF-I and GH conditions. Cells were trypsinized; total RNA was extracted and reverse-transcripted. cDNA for each gene was quantified by real-time quantitative PCR. Relative expression levels of the various transcripts were generated for each one and relative abundance was then determined by comparing the cycle threshold (C_t) values for each reaction with comparative C_t method ($2^{\Delta\Delta Ct}$) with respect to the control condition. Variations in input cDNA mass were corrected by normalizing all data with the corresponding huCYC levels. All measurements were performed in triplicate and in eight independent experiments from eight different fetuses (5 females and 3 males; VitD: vitamin D).
* $p < 0.05$.

and activator of transcription 5b) gene expression (data not shown). No difference in any effect was observed between sexes (data not shown).

Growth plate IGF-I expression and production have been demonstrated to be crucial for longitudinal skeletal growth, although it has been suggested that the effect of GH on the germinal zone may be mediated by local IGF-II production [33]. IGF-I production in growth plate chondrocytes has been questioned until recently, but we demonstrated by real-time quantitative PCR that although IGF-II is the most prominent IGF expressed, chondrocytes from human fetal growth plate expressed IGF-I mRNA [21].

VitD stimulation of IGF-I expression was statistically significant from 10^{-8} M which was confirmed by the IGF-I protein parallel increase in the culture medium. The stimulatory effect of VitD on IGF-I release has been observed *in vitro* in rat chondrocytes [25]; however, conflicting results exist, which suggests that the effect of VitD on IGF-I synthesis and release may be divergent, probably depending on differences in cell types, culture conditions and differentiation status of the cells [34–36].

In our culture system, VitD stimulation of IGFBP-3 was dose-dependent and statistically significant from 10^{-11} to 10^{-6} M. The induction of IGFBP-3 by VitD appeared to be directly mediated via VDR interaction with a putative VDRE (vitamin D-responsive element) between −3296 and −3282 upstream of the human IGFBP-3 gene [37]. Expression of other IGFBPs had not been determined in the present study but results obtained in our chondrocyte culture media showed that although IGFBP-1, IGFBP-2, IGFBP-4 and IGFBP-6 proteins were detectable by immunoassays, levels of IGFBP-3 were significantly higher (data not shown) and that IGFBP-3 production by chondrocytes was higher than that produced by other cell types such as human fetal dermal fibroblasts and the human osteoblast cell line SAOS-2 (data not shown).

In our experimental conditions, we observed that VitD had a variable effect on IGF-IR gene expression: a tendency was observed towards stimulation which was significant at 10^{-6} M (data not shown). This would further contribute to IGFBP3 expression stimulation in response to combined IGF-I and VitD as observed in our results.

Our results demonstrated that VitD strongly stimulated GHR expression from 10^{-10} M in human fetal growth plate chondrocytes and this concurs with the results of Morales et al. [38] which showed that VitD prolonged GH signaling via the JAK2/STAT5 pathway in UMR106 cells and that pretreatment of cells with VitD was necessary to detect GH-induced STAT5 transcriptional response; furthermore, the pretreatment of cells with VitD recovered their capacity to respond to repetitive GH stimulation [38] and this fitted with our results, which showed that VitD strongly stimulated GHR expression.

Our results showed that GH alone at a high concentration had no effect on serum-deprived first-passage human fetal epiphyseal chondrocyte gene expression, although they express GHR, IGF-I and IGF-II. However, when VitD was present with a concentration-dependent intensity, GH was able to further stimulate IGF-I gene expression. We may hypothesize that when GHR mRNA is more abundant in the presence of VitD, GH is able to enhance IGF-I gene expression. In our system, no significant regulation of IGF-II expression was detected either by VitD or by GH, which suggests that IGF-I could contribute more significantly to fetal growth regulation although, as referred to in our results, IGF-II mRNA was more abundant than IGF-I mRNA. Our study was limited to human epiphyseal chondrocytes from fetuses 14–22 weeks old and GH action on postnatal human growth plate chondrocytes could not be anticipated from the present results.

SOX9 has been shown to be required for expression of cartilage-specific marker genes including COL2A1 and aggrecan [39], and as SOX9 was found to bind and activate a specific enhancer element in both COL2A1 [40] and aggrecan [41], the inhibitory effect of VitD on COL2A1 and aggrecan expression could be indirect via SOX9 expression inhibition. In agreement with our results, Horton et al. [42] showed by Northern blot, in an immortalized rat chondrocyte cell line, that VitD inhibited aggrecan expression (10^{-8} M resulted in a 90% decrease in aggrecan mRNA). These results suggest that the effect of VitD on human growth plate is accompanied by decreased expression of specific transcription factors and proliferative chondrocyte matrix proteins, such as SOX9, COL2A1 and aggrecan and by stimulated COMP, which was found to be low in the growth plate proliferative zone and the cessation of proliferation, coincided with its maximum expression pattern [43].

The effects of vitamin D deficiency on fetal and postnatal human skeletal growth have been reported in nutritional deficiency and VitD resistance. The growth plate of vitamin D-deficient rickets patients showed an increased and disorganized hypertrophic zone and diminished mineralization of the lowest metaphyseal zone; patients showed linear growth retardation although bone bowing hinders measurement accuracy. Vitamin D supplementation and rickets cure afford accelerated growth and increased IGF-I serum levels [8]: whether this increase in circulating IGF-I is secondary to increased GH secretion or, at least in part, to increased local IGF-I production as stimulated by VitD remains to be elucidated. Although normal size at birth has been described in hereditary VitD-resistant (rickets type II) children, probably due to transplacental calcium transfer, Laufer et al. [44] reported that these children are often growth-retarded; although GH secretion is normal [45] and normal growth is restored by massive calcium administration. Moreover, low vitamin D levels in late pregnancy have recently been associated with reduced intrauterine bone growth [46].

5. Conclusions

In summary, our results demonstrate that VitD is a potent inhibitor of human fetal epiphyseal growth plate chondrocyte proliferation and that it inhibits expression of several genes related to proliferative chondrocytic phenotype and stimulates expression of several GH–IGF axis genes such as IGF-I, IGFBP-3 and GHR. Our results suggest that VitD regulation of fetal growth plate could consist of parallel enhancing of cell differentiation and conditioning to a phenotype more sensitive to regulation by other hormones such as GH as shown by increased GHR and IGF-I expression.

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