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Severity of experimental autoimmune encephalomyelitis is unexpectedly reduced in mice born to vitamin D-deficient mothers☆

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ABSTRACT

Accumulating data indicate that vitamin D, a sun-induced hormone, plays a key role in multiple sclerosis (MS) etiology. Notably, it has been shown that there is a remarkable season of birth effect in MS. We surmised that gestational vitamin D deficiency is a risk factor for MS. To test this hypothesis, a vitamin D deficiency was induced in C57BL/6 female mice 6 weeks prior to conception and prolonged until offspring birth. Contrary to our prediction, we show here that adult offspring exposed to developmental vitamin D deficiency (DVD) developed a striking milder and delayed experimental autoimmune encephalomyelitis (EAE), when compared to control offspring. Using reverse transcription and quantitative real-time PCR, we measured the expression level of 22 candidate transcripts in the spleen, the cerebrum and the spinal cord, at Day_0 and Day_{30} post-immunization. We report here that, at Day_{30} post-immunization, *TNF*, *osteopontin*, *H2-Eb* were over-expressed and *IFN* was under-expressed in the spinal cord of control mice and not in DVD mice. Another discrepancy between nervous and immune systems was observed: expression of *IL4* was dysregulated exclusively in the spleen. Reduced symptom severity in DVD mice can partially be explained by a nervous system-restricted over-expression of vitamin D receptor (*VDR*), two heat shock proteins (*HSP90*, *HSPa8*) and FK506 binding protein 1a (*FKBP1a*), at Day_0. Our clinical test and molecular findings converge to indicate that maternal hypovitaminosis D imprints the foetus and alters the susceptibility of the offspring to EAE. We propose a new hypothesis to explain our unexpected observations.

1. Introduction

The complex etiology of Multiple Sclerosis (MS) remains unclear and both genetic and environmental factors contribute to MS risk [1,2]. To date, the two most striking environmental clues remain the latitude gradient and the month of birth effect. On the one hand, it has been repeatedly found that prevalence of MS augments with increasing latitude, worldwide and within countries (for a review [3]). On the other hand, MS prevalence is influenced by birth date. In northern countries, the risk of developing MS is lower for those born in November [4,5]. These data suggest that an environmental factor operating during gestation and/or the neonatal period is a candidate risk-modifying factor for MS. Since latitude and month of birth are related to sunlight exposure, and sunlight supports vitamin D synthesis [6], others and we hypothesized that low vitamin D status during gestation is a risk factor for MS.

In order to test this hypothesis, we devised a developmental vitamin D deficiency (DVD) model [7,8]. The adult DVD offspring underwent an EAE induction and were compared to controls. In addition, we extracted total RNAs in three different tissues (spinal cord, cerebrum, spleen) and measured the expression level of 22 candidate transcripts by reverse transcription and quantitative real-time PCR (qRT-PCR). The candidate transcripts were chosen considering their role in (i) vitamin D metabolism; (ii) EAE pathology [9]; (iii) myelination; and (iv) results from two previous pan-genomic and pan-proteomic studies, in which we described that permanent changes are induced in a rat DVD model [10,11].

2. Materials and methods

2.1. Animal housing and feeding

All procedures were performed according to the French law on Animal Care Guidelines. Animal Care Committee of University Aix-Marseille II approved protocols. C57Bl/6 mice (Charles River, France) were maintained in a holding room at a constant temperature of $21 \pm 2\,^{\circ}C$ and 60% relative humidity, on a 12 h light–dark cycle. Food and water were provided *ad libitum*. Vitamin D deficiency was achieved by (i) feeding fertile adult female mice with either a normo-calcemic and normo-phosphatic vitamin D_3-free diet (INRA, France) or a normo-phosphatic vitamin D3-free diet supplemented with lactose and calcium (INRA, France) and (ii)

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using UV-free lighting. Control animals (males and females) were given a standard vitamin D_3-containing (1500 IU/kg) diet (INRA, France). Serum vitamin D depletion was assessed 6 weeks later using a commercial RIA (Diasorin, MN, USA) for 25-hydroxyvitamin D_3. Dams exposed to 6 weeks of vitamin D_3 depletion exhibited a reduced production of 25-hydroxyvitamin D (mean of 3 ng/ml ± 0.5) when compared to control dams (40 ng/ml ± 2.5). Vitamin D-deficient females were then mated with control males and kept under vitamin D_3-free conditions throughout gestation. At birth, offspring (DVD or DVD supplemented with calcium and lactose mice) and dams were placed on the vitamin D_3-containing control diet. In parallel, control females were mated with control males in order to obtain control offspring (control mice). Offspring were weaned 28 days after birth and were kept with the control diet for the rest of their life.

2.2. Active MOG35-55-induced EAE

12-week-old female offspring were immunized subcutaneously with 250 μg of 35-55 MOG peptide (sequence: MEVGWYRSPFSRVVHLYRNGK, Genepep, France), emulsified in complete Freund adjuvant (DIFCO, USA) and supplemented with 400 μg of H37Ra *Mycobacterium tuberculosis* (DIFCO, USA). 100 ng of pertussis toxin was injected ip, at Day_0 and Day_1 post-immunization. Immunized females were randomly placed in different cages. Weight and disease severity were blindly scored, once a day, according to the EAE clinical scale: 0 = no detectable sign of EAE; 1 = weakness of the tail; 2 = tail paralysis and hind limb weakness; 3 = partial paralysis of hind limbs; 4 = complete paralysis of hind limbs; 5 = complete paralysis of hind limbs with incontinence and partial or complete paralysis of forelimbs; 6 = dead, as previously described [12].

2.3. Tissue collection, RNA isolation and reverse transcription

After deep anesthesia, cerebra, spinal cords and spleens were dissected from either 12 or 16-week old DVD females and control females, at the time points corresponding to Day_0 and Day_{30} post-immunization. Tissues were immediately flash-frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated from all single tissues using RNeasy kit with the DNAse on-column treatment (Qiagen, France), according to the manufacturer's instructions. cDNAs were obtained after reverse transcription of each total RNA with SuperScript III and oligo(dT) primers (Invitrogen, USA).

2.4. Design of primers and transcriptional assessment of candidate genes

Candidate genes were chosen according to previous results of DVD model microarrays [10,11], as well as pathological features of EAE. GAPDH and *HPRT1* were used as reference genes. Primers of each candidate gene (supplementary Table 1) were designed using Primer 3 on-line free software (http://frodo.wi.mit.edu) and were synthesized by Eurofins MWG Operon (Germany).

Quantitative real-time PCR (qPCR) was carried out with the Light Cycler® 480 apparatus (Roche Applied Science, France). Reactions were performed in duplicate and set up in a total volume of 20 μl containing 50 ng of cDNA template, 10 μl of Light Cycler® 480 SYBR Green I Master Mix and each pair of primers at a final concentration of 0.2 μM. The general amplification conditions were a pre-incubation at 95 °C for 5 min, 45 cycles of 95 °C for 10 s, 67 °C for 15 s, 72 °C for 15 s of amplification; a melting curve and a final cooling at 40 °C for 10 s. Threshold cycle values (Ct) of the target and the reference genes as well as the final results for each sample were automatically calculated by the Light Cycler 480 software (ΔCt method). *GAPDH* or *HPRT1* were chosen for normalization according to their Ct. The housekeeping gene whose expression was the closest to the Ct of the candidate gene was elected for comparison.

2.5. Statistical analysis

EAE and qPCR data were analyzed using parametric (ANOVA) and non-parametric (Mann–Whitney) tests and GraphPad Prism software. In all analyses, $P < 0.05$ was selected as threshold for significance.

3. Results and discussion

3.1. Developmental vitamin D deficiency delays the onset of EAE and reduces symptom severity in adult offspring

The three tested groups developed MOG35-55-induced EAE (Fig. 1). However, developmental vitamin D deficiency significantly altered EAE course. Both DVD and DVD supplemented with calcium and lactose mice developed a significantly delayed EAE onset (onset day 17.7 ± 1.1 and 15.4 ± 0.5, respectively) when compared to controls (onset day 13.2 ± 0.4, $F = 8.6$, $p = 0.0011$). Moreover, both DVD and DVD supplemented with calcium and lactose mice developed a reduced clinical score peak (1.5 ± 0.3 and 1.5 ± 0.4, respectively) when compared to controls (3.8 ± 0.1, $F = 5.95$, $p = 0.0066$). At Day_0 post-immunization, the mean weight of the three tested groups was similar (DVD: 20.9 ± 0.9 g; DVD supplemented with calcium and lactose mice: 20.0 ± 0.6 g; controls: 19.6 ± 0.4 g). DVD and DVD supplemented with calcium and lactose mice exhibited similar clinical parameters. This finding allowed us to exclude an alteration of calcium/phosphorus homeostasis as a causal factor. To further investigate the mechanism underlying the delayed and milder EAE, the expression level of 22 candidate transcripts was measured in DVD and control mice.

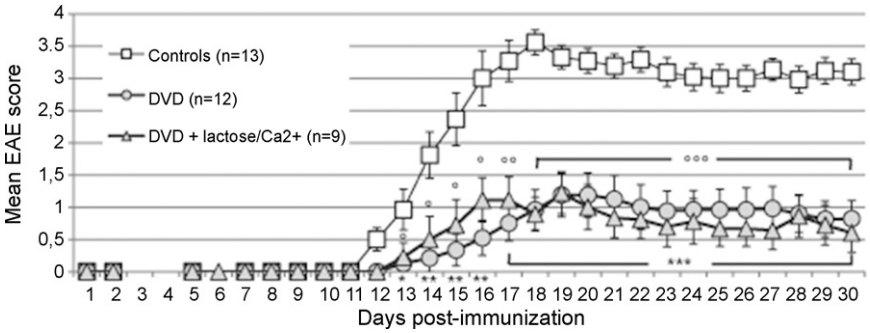

Fig. 1. Clinical course of MOG35-55-induced EAE in C57Bl/6J mice. DVD mice, whose mother was fed with a vitamin D-free diet (circle) or with a vitamin D-free diet supplemented with calcium and lactose (triangle), display a delayed onset and less severe symptoms when compared to control mice (square). Error bars indicate SEM. Significant differences for mean EAE scores are reported with either degree symbol (DVD to control mice) or asterisk (DVD + lactose/calcium to control mice). °/*$p < 0.05$, °°/**$p < 0.005$, °°°/***$p < 0.0001$.

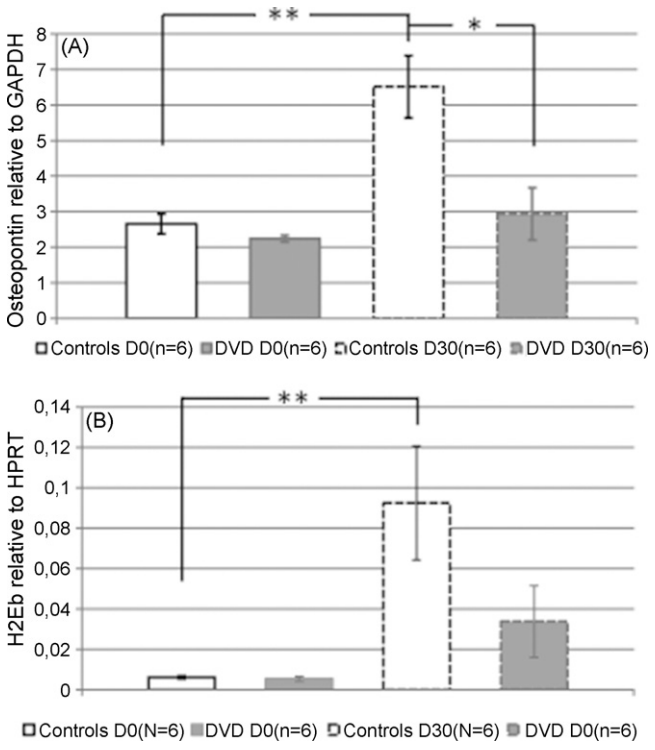

Fig. 2. Transcripts of *osteopontin* and *H2-Eb* are over-expressed in control mice at Day_{30} post-immunization. Spinal cords from control and DVD mice were collected at Day_0 (D_0) or at Day_{30} post-immunization (D_{30}), total mRNA extracted and expression of genes coding for osteopontin (A) and H2-Eb (B) measured by quantitative PCR. Control mice display an increased expression of the two transcripts at D_{30} while DVD mice exhibit a null or a mild increased production. Error bars indicate SEM. Significant differences for mean values are reported with $^{*}p < 0.05$; $^{**}p < 0.005$.

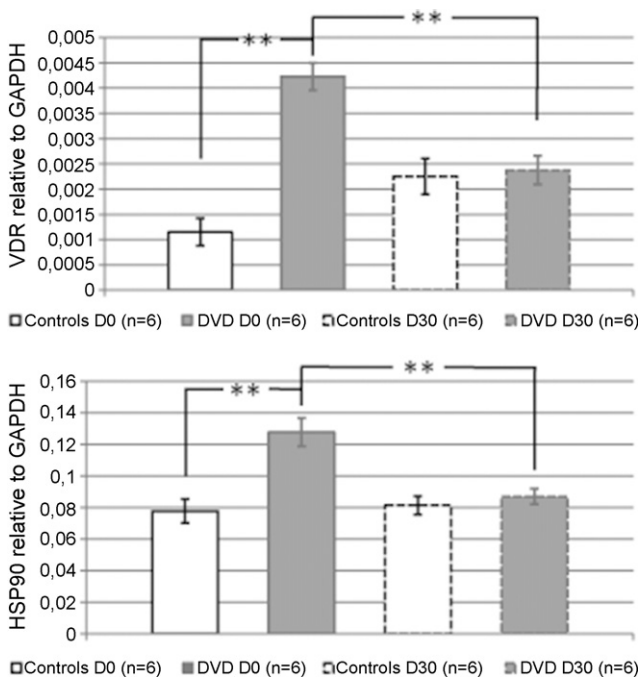

Fig. 3. Transcripts of *VDR* and *HSP90* are over-expressed in DVD mice at Day_0. At Day_0 (D_0), before the EAE induction, *VDR* (A) and *HSP90* (B) are strongly over-expressed in spinal cord of DVD mice. The induction of EAE down-regulated the expression of both transcripts in the spinal cord of DVD mice. Error bars indicate SEM. Significant differences for mean values are reported with $^{*}p < 0.05$; $^{**}p < 0.005$.

3.2. DVD mice exhibit a reduced inflammatory response at Day_{30} post-immunization

At Day_{30} post-immunization, an over-expression of *MOG* transcripts was observed in the spinal cord of control mice and within the cerebrum of DVD mice (supplementary Table 2). This result likely reflects a compensatory repair mechanism dedicated to compensate MOG35-55-induced demyelination [13]. Significant variations were observed in the central nervous system at Day_{30} post-immunization. When compared to control mice at Day_0, two pro-inflammatory factors, *TNFalpha* and *osteopontin*, as well as the *H2-Eb* (mice homolog of *HLA-DRB1*) were over-expressed at Day_{30} post-immunization in the spinal cord of control mice (supplementary Table 2 and Fig. 2). Such a significant increase was not observed in DVD mice between Day_0 and Day_{30}. Interestingly, at Day_{30} post-immunization, a significantly reduced expression of *IFN*, a cytokine known for repressing EAE [14,15], was observed in the spinal cord of control mice. Both groups displayed an increased expression of *IL1*, a pro-inflammatory cytokine, but the over-expression was lesser in the DVD group (supplementary Table 2). An over-expression of *IL23*, another pro-inflammatory cytokine, was measured in the spinal cord and the cerebrum of DVD mice (supplementary Table 2). In our conditions, expression of *IL17* was not quantifiable. Within the spleen, only *IL4*, an anti-inflammatory cytokine, exhibited an increased expression in DVD mice. This finding is in accordance with a previous study showing that vitamin D up-regulates IL4 expression [16]. Overall, molecular data are strongly correlated to the clinical course of EAE.

3.3. DVD mice exhibit an up-regulation of VDR, Hspa8, HSP90 and Fkbp1a before EAE induction

At Day_0, VDR was strongly over-expressed within the spinal cord of DVD mice, when compared to control mice (supplementary Table 2 and Fig. 3). This result is in line with our data on MOG expression: a reduced transcript expression of this molecule, reflecting probably a lowered demyelination, is found in the area of the central nervous system where VDR is up-regulated. Importantly, it should be emphasized that the immunosuppressive properties of vitamin D in EAE is mediated by the VDR [17]. Similarly, *HSP90*, *Hspa8* and *Fkbp1a* were over-expressed at Day_0 within the spinal cord of DVD mice, when compared to control mice (supplementary Table 2). Heat Shock Proteins (HSP) are known mediators of VDR action [18–20]. We show here for the first time that HSPs may also be involved in vitamin D-induced immuno-regulation. In addition, the overexpression of *Fkbp1a*, a chaperone molecule that plays a pivotal role in immunosuppression [21], correlates with the delayed and mild EAE score. At Day_{30} post-immunization, DVD mice exhibited a down-regulation of *VDR*, *Hspa5*, *Hspa8*, *HSP90* and *MARRS*, when compared to DVD mice at Day_0 (supplementary Table 2). MARRS, a putative vitamin D membrane receptor, also known as GRP58/ERp57, interacts with MHC class I molecules and therefore may play an unrecognized role in EAE response [22].

We bring here evidence that a transient prenatal hypovitaminosis D imprints the developing offspring and induces an altered response of the immune system after immunization. In addition, we show for the first time that Vitamin D receptor and Heat Shock Proteins may mediate, at least partially, this modulation. However, one unexpected finding – the resistance of DVD mice to EAE – remains to be elucidated. This paradoxical response might be explained if we postulate that our gestational vitamin D deficiency induces, *via* an overexpression of the VDR and an increased sensitivity to the ligand, a post-natal vitamin D supplementation. We reason that, during pregnancy, mouse embryos were developing in

a vitamin D-deficient environment and when, at birth, newborns were fed with adequate levels of vitamin D, they grew up in an enriched-like vitamin D environment. Further experiments, including post-natal vitamin D supplementation, need to be performed in order to (un)validate this new hypothesis which is supported by epidemiological studies showing that a reduced risk of MS is associated with higher sun exposure for children in Tasmania when aged 6–15 years [23] or summer outdoor activities in childhood and adolescence in Norway [24].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2010.03.006.

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