Expression of the Multiple Sclerosis-Associated MHC Class II Allele HLA-DRB1*1501 Is Regulated by Vitamin D

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Abstract

Multiple sclerosis (MS) is a complex trait in which allelic variation in the MHC class II region exerts the single strongest effect on genetic risk. Epidemiological data in MS provide strong evidence that environmental factors act at a population level to influence the unusual geographical distribution of this disease. Growing evidence implicates sunlight or vitamin D as a key environmental factor in aetiology. We hypothesised that this environmental candidate might interact with inherited factors and sought responsive regulatory elements in the MHC class II region. Sequence analysis localised a single MHC vitamin D response element (VDRE) to the promoter region of HLA-DRB1. Sequencing of this promoter in greater than 1,000 chromosomes from HLA-DRB1 homozygotes showed absolute conservation of this putative VDRE on HLA-DRB1*15 haplotypes. In contrast, there was striking variation among non–MS-associated haplotypes. Electrophoretic mobility shift assays showed specific recruitment of vitamin D receptor to the VDRE in the HLA-DRB1*15 promoter, confirmed by chromatin immunoprecipitation experiments using lymphoblastoid cells homozygous for HLA-DRB1*15. Transient transfection using a luciferase reporter assay showed a functional role for this VDRE. B cells transiently transfected with the HLA-DRB1*15 gene promoter showed increased expression on stimulation with 1,25-dihydroxyvitamin D3 (P = 0.002) that was lost both on deletion of the VDRE or with the homologous “VDRE” sequence found in non–MS-associated HLA-DRB1 haplotypes. Flow cytometric analysis showed a specific increase in the cell surface expression of HLA-DRB1 upon addition of vitamin D only in HLA-DRB1*15 bearing lymphoblastoid cells. This study further implicates vitamin D as a strong environmental candidate in MS by demonstrating direct functional interaction with the major locus determining genetic susceptibility. These findings support a connection between the main epidemiological and genetic features of this disease with major practical implications for studies of disease mechanism and prevention.

Introduction

Multiple sclerosis (MS) is a common inflammatory disease of the central nervous system characterized by myelin loss, axonal pathology, and progressive neurological dysfunction [1]. The aetiology of MS is unknown, however it is clear that genetic and environmental components are important [1,2].

The only genetic association with MS in Northern Europeans had been with extended MHC haplotypes, especially those containing HLA-DRB1*1501 [3]. The interleukin 7 receptor (IL7RA), interleukin 2 receptor (IL2RA), ectotropic viral integration site 5 (EVI5) and kinesin family member 1B (KIF1B) genes have recently been shown to be additional MS susceptibility loci [4,5,6,7]. The largest of these, KIF1B, has a relatively small effect size (odds ratio (OR) = 1.3). The MHC (OR = 5.4) is the key susceptibility locus in MS and other susceptibility genes identified to date appear to contribute little to overall risk [3].

The principal MHC class II haplotype that increases MS risk in individuals of Northern European descent is HLA-DQB1*0602-DQA1*0102 -DRB1*1501-DRB5*0101 [8], although other HLA-DRB1 haplotypes have important influences on risk by epistatic interactions [9,10,11,12]. Intense linkage disequilibrium within the MHC has frustrated attempts at fine mapping and no precise susceptibility locus has been identified [9,13].

Twin studies have established that monozygotic (MZ) twin concordance is significantly greater than for dizygotic (DZ). In the study by Wiirer and colleagues concordance was 25.3% and 5.4% respectively [14]. The observation that most MZ twin pairs are discordant for MS suggests environmental, stochastic factors or both but the most striking illustration of the importance of the...
Multiple Sclerosis (MS) is a complex neurological disease with a strong genetic component. The Major Histocompatibility Complex (MHC) on chromosome 6 exerts the strongest genetic effect on disease risk. A region at or near the HLA-DRB1 locus in the MHC influences the risk of MS. HLA-DRB1 has over 400 different alleles. The dominant haplotype of Northern Europe, marked by the presence of DRB1*1501, increases risk of MS by 3-fold. The environment also plays a key role in MS. The most striking illustration of this is the geographical distribution of the disease in populations matched for ethnicity. This has led to the proposal that sunshine, and in particular, vitamin D, is an environmental factor influencing the risk of MS. Circumstantial evidence supporting this comes from studies showing the involvement of vitamin D in immune and nervous system function. The current investigation sought to uncover any relationship between vitamin D and HLA-DRB1. It was found that vitamin D specifically interacts with HLA-DRB1*1501 to influence its expression. This study therefore provides more direct support for the already strong epidemiological evidence implicating sunlight and vitamin D in the determination of MS risk, and implies that vitamin D supplementation at critical time periods may be key to disease prevention.

Author Summary

Vitamin D is a secosteroid hormone synthesized in the skin or ingested in the diet. Intake from dietary sources accounts for a much smaller proportion of total vitamin D, mainly owing to its rarity in foods [22,23]. During exposure to sunlight, ultraviolet B (UVB) radiation (290–315 nm) is responsible for photolyzing 7-dehydrocholesterol, the precursor of vitamin D3, to previtamin D3 (UVB) radiation (290–315 nm) is responsible for photolyzing 7-dehydrocholesterol, the precursor of vitamin D3, to previtamin D3, which, in turn, rapidly spontaneously isomerizes to vitamin D3 [22,23]. Vitamin D3 is biologically inert and requires hydroxylation in the liver to 25-hydroxyvitamin D3 (25(OH)D). Once formed, this major circulating form of vitamin D3 is further hydroxylated in the kidney to its active form, 1,25-dihydroxyvitamin D3 (1,25(OH)2D), by 25-hydroxyvitamin D-1α-hydroxylase (1-OHase). Recently it has been recognized that most tissues in the body (including the brain, thymus and cells of the immune system) also possess the 1-OHase enzyme. Thus numerous tissues in the body have the capacity to locally produce 1,25(OH)2D [22,23].

Most biological effects of 1,25-dihydroxyvitamin D3 or calcitriol, are mediated by the vitamin D receptor (VDR). This receptor is a member of the steroid receptor super-family and influences the rate of transcription of vitamin D responsive genes by acting as a ligand activated transcription factor that binds to vitamin D response elements (VDREs) in gene promoters [21]. Early studies had provided evidence for an effect of vitamin D on HLA gene expression [24,25], although no specific mechanism has been characterised. Here we examined the hypothesis of a direct interaction between vitamin D and MS associated MHC class II genes. Genetic variation characteristic of the most significant risk haplotypes for MS, those bearing HLA-DRB1*15, includes a functional vitamin D response element (VDRE) in the proximal promoter region of HLA-DRB1. This provides a mechanism linking the major environmental and genetic risk factors for MS.

In Silico Identification of Putative Vitamin D Response Elements

Using the sequence for the HLA-DRB1*15 haplotype carried by the homozygous lymphoblastoid cell line PGF we scanned in silico for VDREs using Jaspar [26] with a profile score threshold of 80%. We analysed the entire genomic sequence of the HLA-DRB1, HLA-DQA1 and HLA-DQB1 genes as well as 5 kb upstream of the transcriptional start sites of these genes to include promoter regions. VDREs exhibit a multitude of sequence variations, providing a spectrum of binding affinities for VDR, thus enabling these elements to respond to differing concentrations of VDR/1,25(OH)2D [22]. The analysis revealed only one potential VDRE located in the proximal promoter region immediately 5’ to the transcriptional start site of HLA-DRB1 (Figure 1). IL2RA and IL7RA were also searched in silico for potential VDR binding sequences; no putative VDREs were found.

Sequencing of the HLA-DRB1 Promoter in MS Patients and Controls

The occurrence and conservation of the putative VDRE element identified in the PGF sequence was examined in individuals with the HLA-DRB1*15 MS risk allele. The HLA-DRB1 promoter was resequenced in 322 HLA-DRB1*15 homozygous individuals, both MS affected and unaffected. An additional 168 individuals homozygous for other HLA-DRB1 alleles were also sequenced. The putative VDRE was present on all HLA-DRB1*15 bearing haplotypes with no variants found which disrupted the VDRE consensus sequence. In contrast, a number of nucleotide changes were found within the 15 base pairs of the VDRE on non-HLA-DRB1*15 haplotypes. For example, nearly all (98% of 57 sequenced individuals) of HLA-DRB1*04, HLA-DRB1*07 and HLA-DRB1*09 haplotypes, all of which are non-MS associated alleles in the Canadian population [10], carried the sequence GGGTGGAGAGGGGTCA. This sequence was predicted to function less effectively as a VDRE than the one on HLA-DRB1*15 bearing haplotypes according to Jaspar [26]. The modestly MS associated haplotype, HLA-DRB1*17, differed from HLA-DRB1*15 at the VDRE in 50% of the individuals sequenced.

In Vitro Binding of VDR to the HLA-DRB1*15 VDRE

The putative VDRE in the HLA-DRB1 promoter was investigated for ability to bind the vitamin D receptor in vitro using an electrophoretic mobility shift assay (EMSA). Upon addition of recombinant VDR and retinoic acid receptor beta (RXR), a co-regulator of VDR binding and transactivation [22] to a radiolabelled probe spanning the putative VDRE in the HLA-DRB1 promoter, two protein-DNA complexes on EMSA were observed (Figure 2, lane 2). Both complexes were specifically...
competed with 10 to 100-fold molar excess of unlabelled VDRE probe (Figure 2, lanes 3–5), while 10 to 100 fold molar excess of an unrelated probe containing an early growth response (EGR) factor binding site had no effect (Figure 2, lanes 6–7). Finally, addition of a polyclonal antibody directed against VDR specifically retarded complex I, resulting in a supershift of the upper complex (Figure 2, lane 8). This data showed the putative VDRE in the **HLA-DRB1** promoter corresponding to the **HLA-DRB1*15** haplotype could bind recombinant VDR/RXR with high specificity *in vitro*. When probes corresponding to the **HLA-DRB1*04/07/09** variant VDRE were used, significantly lower affinity binding was found (*data not shown*).

**Evidence Ex Vivo of VDR Binding to the VDRE Found on the HLA-DRB1*15 Haplotype**

Whether or not the VDR is recruited to the VDRE in the **HLA-DRB1** gene promoter was examined *ex vivo*. Chromatin immunoprecipitation (ChIP) experiments were performed using lymphoblastoid cells bearing the **HLA-DRB1*15** haplotype (the PGF cell line) which were either unstimulated or stimulated for 24 hours with 1,25-dihydroxyvitamin D3 and then cross-linked in the presence of formaldehyde. Immunoprecipitation was performed using antibodies against VDR. The VDR bound DNA fragments were then recovered after reversal of protein-DNA crosslinking and analysed by PCR using primers specific for the **HLA-DRB1** promoter. A representative agarose gel is shown in Figure 3. This revealed clear evidence of binding by VDR to the **HLA-DRB1** promoter when compared to input chromatin and mock antibody controls for cells with the **HLA-DRB1*15** haplotype, complementing the *in vitro* data from the EMSA experiments.

**Transient Transfection**

The VDRE was then investigated to see if it modulated levels of gene expression *in vitro*. Reporter gene constructs were engineered in which −181 to +53 of the **HLA-DRB1** gene sequence was placed upstream of a pGL3 luciferase reporter. pGL3_DRB1prom had the complete −181 to +53 sequence, pGL3_DRB1prom_hap1 had the same sequence as pGL3_DRB1prom but the VDRE replaced with the **HLA-DRB1*04/07/09** VDRE, and pGL3_DRB1prom_del had the 15 base pair VDRE sequence specifically deleted. These constructs were then transiently transfected into Raji B cells. A renilla luciferase reporter construct driven by the thymidine kinase promoter (pRL_TK) was co-transfected to normalise luciferase activity. pGL3_DRB1prom had significantly higher basal reporter gene activity than pGL3_DRB1prom_del (\(P=0.03\) on paired *t*-test, two tailed). After stimulation with 1,25-dihydroxyvitamin D3, there was a significant 1.6 fold increase in luciferase activity with
Flow Cytometry

To investigate any effect of vitamin D on the cell surface expression of HLA-DRB1, the HLA-DRB1*15 homozygous lymphoblastoid cell line PGF and the HLA-DRB1*07 homozygous lymphoblastoid DBB cell line were stained with anti-HLA-DRB1 antibody. PGF cells constitutively expressed HLA-DRB1 at higher levels than DBB (average geometric mean fluorescence intensity (MFI) PGF = 97.1, DBB = 42.8, \( P = 0.0002 \)). Upon addition of 1,25-dihydroxyvitamin D3, there was a 1.3 fold increase in the expression of HLA-DRB1 in PGF cells (\( P = 0.031 \) on paired \( t \)-test, two tailed) but no significant difference in the expression of HLA-DRB1 in DBB cells (\( P = 0.10 \)).

Discussion

While the role of the environment is clearly important in determining MS risk, the relevant underlying mechanism(s) have remained elusive and there has been no experimental support for a direct environment-gene interaction. Although differences in Epstein-Barr virus infection are seen when MS patients are compared to controls, extensive searches for specific viral infections have failed to confirm direct involvement [2]. Where appropriate data is available, the amount of winter sunlight parallels the range of MS prevalence, and high sunlight exposure is associated with low disease prevalence [2]. The effects of migration between high and low risk geographic regions have been examined in several populations (e.g. UK immigrants to South Africa, or Asian and Caribbean immigrants to the UK). These studies show that MS risk is influenced by the migrant’s country of origin [27]. Despite the limits of small sample sizes, a ‘critical age’ has been hypothesized: immigrants who migrate before adolescence acquire the risk of their new country, while those who migrate after retain the risk of their home country. Dietary difference for vitamin D intake (oily fish consumption) plausibly explains the striking exception to MS latitudinal risk in Norway [2]. As familial aggregation is genetically determined [28], environmental factors thus appear to be operative at a broad population level, perhaps acting at a young age [27] and/or during gestation [20]. A good candidate for an environmental factor that influences MS disease risk is vitamin D.

We approached the candidacy of vitamin D by searching first for vitamin D response elements within the MHC class II region. Specifically we investigated the major candidate genes in the disease associated locus, HLA-DRB1, HLA-DQA1 and HLA-DQB1 and identified a consensus binding site for VDR next to the HLA-DRB1 gene. This was the only VDRE we found and strikingly it shows haplotype-specific differences, being highly conserved in the major MS associated haplotype HLA-DRB1*15 dominant in Northern European populations, but not conserved among non-MS associated haplotypes. This was itself circumstantial evidence supporting a vitamin D role in the functional characteristics of this haplotype. The identified VDRE lies close to the highly conserved MHC class II specific regulatory SXY module. This module comprises S, X and Y regulatory elements important for constitutive, and indirectly for IFN-γ-induced, expression of HLA class II genes co-ordinated by the MHC class II transactivator MHC2TA [29]. The VDRE was highly conserved on HLA-DRB1*15 haplotypes (no mutations on over 600 chromosomes) suggesting a selective pressure to maintain this response element for the HLA-DRB1*15 allele. Variants were found to some extent on all other non HLA-DRB1*15 haplotypes. The results may additionally/alternatively reflect the ancestral
origin of the HLA-DRB1*15 (DR51) haplotype [30] which displays the strongest linkage disequilibrium among the MHC class II haplotypes [31]. We note the association between this haplotype and MS risk is characteristic of Northern European populations, the ones most vulnerable to vitamin D deficiency [2].

EMSA experiments using recombinant proteins demonstrated that in vitro VDR can bind specifically to the putative VDRE in the proximal HLA-DRB1 promoter found on the HLA-DRB1*15 haplotype. ChiP data showed specific enrichment of the region spanning the VDRE in VDR immunoprecipitated samples relative to input and mock antibody controls, demonstrating that the vitamin D receptor was recruited to this haplotype in this in vivo model system. Finally, transient transfection and flow cytometric assays established that the VDRE present in the HLA-DRB1 promoter can influence gene expression and imparts 1,25-dihydroxyvitamin D3 sensitivity to HLA-DRB1*15. The variant VDRE present on other, non-MS associated HLA-DRB1 haplotypes was not responsive to 1,25-dihydroxyvitamin D3.

A T cell repertoire with millions of specificities provides surveillance against a multitude of foreign pathogens [32]. An inherent danger in recognizing so many foreign proteins is the potential to respond to self-proteins. To circumvent this problem T cells are scrutinised for self-reactivity as they mature in the thymus with deletion of those posing the greatest threat (central deletion) [32]. One constraint on central deletion is the requirement for the relevant autoantigen to be present in the thymus. Whether or not these are expressed as proteins at levels sufficient to induce T cell deletion is not clear. Given the results of this study, variable expression of HLA-DRB1 could affect central deletion of autoreactive T cells. It is plausible that a lack of vitamin D in utero or early childhood could affect the expression of HLA-DRB1 in the thymus, and impacting on central deletion. For MS, in HLA-DRB1*15 bearing individuals, a lack of vitamin D during early life could allow auto reactive T cells to escape thymic deletion and thus increase autoimmune disease risk. Indeed it has been shown that antigen presentation in the thymus of VDR knock-out mice is impaired [33]. However the mechanism for a HLA- vitamin D interaction remains unclear as is the timing and tissue in which such interactions might occur. A major selective pressure on skin pigmentation is thought to have been vitamin D deficiency with progressively lighter skin pigmentation at increasing distance from the equator related to variation in intensity of ultraviolet radiation with latitude [34]. The presence of a VDRE specific to HLA-DRB1*15- bearing haplotypes, present at high allele frequencies among Northern Europeans, suggests a possible role for vitamin D in selection at this locus. The intriguing possibility that vitamin D responsiveness rather than any antigen-specificity determines the increased MS risk of the HLA-DRB1*15 haplotype warrants consideration and can be tested in the infrequent haplotypes bearing the VDRE on other non-HLA-DRB1*15 haplotypes.

In summary, we have identified and functionally characterised a vitamin D response element (VDRE) in the HLA-DRB1 promoter region. These studies imply direct interactions between HLA-DRB1, the main susceptibility locus for MS, and vitamin D, a strong candidate for mediating the environmental effect. This study provides more direct support for the already strong epidemiological evidence implicating sunlight and vitamin D in the determination of MS risk. Given that a high frequency of vitamin D insufficiency in the general population has been observed [35], our data support the case for supplementation during critical time periods to reduce the prevalence of this devastating disease.

Materials and Methods

Subjects, HLA-DRB1 Genotyping, Sequencing

All participants in the study were ascertained through the ongoing Canadian Collaborative Project on the Genetic Susceptibility to MS (CCPGSMS) [36]. Subject ascertainment, genotyping and sequencing has been previously described [9,10,37].

Ethical Statement

Each participating clinic in the CCPGSMS obtained ethical approval from the relevant institutional review board, and the entire project was reviewed and approved by the University of British Columbia and the University of Western Ontario.

EMSA

EMSAs were performed as previously described [38]. The VDRE probe comprised of the annealed sense and antisense strands of the nucleotide sequence agctGTGGGTG-GAGGGGTTCATAG, the EGR probe agctAAATCCCCGGCC CCGCAGGGGA and the VDRE variant probe agctGTGG GTGGAGAGGGGTTCATAG. Full length recombinant purified VDR and recombinant purified RXR beta were purchased from Invitrogen, and polyclonal VDR antibody from Affinity Bioreagents. Radioactivity was quantitated with the Packard Cyclone phosphorimager, and analyzed with Optiquant (Perkin Elmer Life Sciences). Values were compared using the Chi square test.

Chromatin Immunoprecipitation

The lymphoblastoid cell line PGF was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.2 mM L-glutamine at 37°C in 5% humidified CO₂. 60×10⁶ cells were harvested unstimulated or after stimulation with 0.1 uM calcitriol (Sigma). Cells were crosslinked using a 1% formaldehyde buffer for 15 minutes at room temperature, quenched with glycine and chromatin prepared as previously described [39]. Chromatin was sheared by sonication in the presence of 212–300 microns glass beads (Sigma) at 4°C using a double step microtip attached to a Branson 450 Sonifier with coupler (Branson) in 30 second bursts (six pulses at 40%) with the samples cooled on ice for 1 minute between pulses. Sonicated chromatin was then processed and subject to immunoprecipitation as previously described [39] using magnetic ‘Dynabeads M-280’ (Dynal) precoated with anti rabbit (Branson) in 30 second bursts (six pulses at 40%) with the samples cooled on ice for 1 minute between pulses. Sonicated chromatin was then processed and subject to immunoprecipitation as previously described [39] using magnetic ‘Dynabeads M-280’ (Dynal) precoated with anti rabbit IgG to which the primary antibody VDR was bound (Affinity Bioreagents). We followed the buffer used for immunoprecipitation and subsequent washes as described [40]. Following reversal of crosslinks, RNase A and Proteinase K digestion, DNA was extracted using phenol-chloroform and amplified by PCR with separation on a 2.0% agarose gel. The primers used for PCR were: forward- GCAACTGGTTCAAACCTTCC and reverse- GTCCCCAGACAAAGCCAGT. Cycling conditions were: 95°C for 30 seconds; 72°C for 30 seconds; 53°C for 10 minutes; a touchdown of 14 cycles (95°C for 30 seconds; 61°C with −0.5°C per cycle, for 30 seconds; 72°C for 30 seconds); 35 cycles of 95°C for 30 seconds, 53.5°C for 30 seconds, 72°C for 30 seconds; 72°C for 7 minutes.

Cell Transfection and Luciferase Reporter Gene Assay

The plasmids were constructed by inserting the promoter region (–181 to +53) of the human HLA-DRB1 gene (pGL3_DRB1) with the VDRE sequence (chr6:32,665,500–32,665,760), pGL3_DRB1prom_del with the VDRE sequence deleted (chr6:32,665,500–32,665,559 combined with chr6:32,665,575–32,665,760) into the pGL3 reporter plasmid. Two independent plasmid preparations were used in transient transfection experiments for each construct.
Raji B cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.2 mM L-glutamine at 37°C in 5% humidified CO₂. Lipofectamine-LTX and PLUS reagent (Invitrogen) were used for transient transfection of expression constructs, following the manufacturer’s protocol. pRL-TK was co-transfected to normalize for transfection efficiency. Where indicated, cells were stimulated with 0.1 μM calcitriol (Sigma) for 24 hours. Cells were harvested after 24 hours and lysed in 500 μl of 1× lysis buffer (Promega) and analyzed using the Dual-Luciferase reporter assay kit (Promega) and a Turner luminometer model 20 (Promega) following the manufacturer’s protocol. Paired t-tests were used to compare expression values. Each transfection was carried out 12 times in total.

Flow Cytometry

The lymphoblastoid cell lines PGF (International Histocompatibility Workshop number IHW09318) and DBB (IHW09052) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.2 mM L-glutamine at 37°C in 5% humidified CO₂. Cells were stained with either a FITC conjugated human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the HLA-DR and CD4 antigen expression, and antigen presentation by 1,25-dihydroxyvitamin D3. Blood 76: 189–197.

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Author Contributions

Conceived and designed the experiments: ADS GCE JCK. Performed the experiments: SVR NJM MRL SGD BMH JCK. Analyzed the data: Svr NJM GCE JCK. Contributed reagents/materials/analysis tools: MJC ADS. Wrote the paper: Svr NJM GCE JCK.

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