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Vitamin D and Autoimmune Diabetes

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Abstract The biologically active form of vitamin D, $1,25(OH)_2D_3$, is a potent modulator of the immune system as well as a regulator of bone and mineral metabolism. Vitamin D-deficiency in infancy and vitamin D receptor gene polymorphisms may be risk factors for insulin-dependent Diabetes mellitus (IDDM). $1,25(OH)_2D_3$ and its analogs significantly repress the development of insulitis and diabetes in the non-obese diabetic (NOD) mouse, a model of human IDDM. $1,25(OH)_2D_3$ may modulate IDDM disease pathogenesis by repression of type I cytokines, inhibition of dendritic cell maturation, and upregulation of regulatory T cells. The function of vitamin D as a genetic and environmental determining factor for IDDM, the protective role of $1,25(OH)_2D_3$ and its analogs in a mouse model of IDDM, and the possible mechanisms by which this protection occurs will be reviewed. J. Cell. Biochem. 88: 216–222, 2003.

Key words: IDDM; NOD mouse; Th1 cells; dendritic cells

Vitamin D has been well characterized as a regulator of bone and mineral metabolism. 1α,25-dihydroxyvitmain D_3 ($1,25(OH)_2D_3$) is the hormonally active form of vitamin D and exert its actions on target tissues through binding to the vitamin D receptor (VDR) [Strugnell and DeLuca, 1997]. Upon ligand binding, this nuclear hormone receptor, in conjunction with its heterodimeric partner, the retinoid X receptor (RXR), regulates gene transcription through vitamin D responsive elements (VDRE) in the promoter regions of vitamin D target genes [Jones et al., 1998]. The circulating level of $1,25(OH)_2D_3$ is tightly regulated, and its formation results from two successive hydroxylations of its precursor, vitamin D_3. Vitamin D_3 can either be obtained through the diet, or from the photochemical conversion of 7-dehydrocholesterol in the skin.

$1,25(OH)_2D_3$ is now widely recognized as a regulator of the immune system in addition to its classical actions on mineral homeostasis. The prospect of vitamin D as an immunomodulatory agent was initiated with the discovery of VDR in peripheral blood monocytes and activated T cells [Bhalla et al., 1983; Provvedini et al., 1983]. Studies in animal models have revealed that $1,25(OH)_2D_3$ is protective against autoimmue diseases including experimental autoimmune encephaomyelitis (EAE) [Lemire and Archer, 1991; Cantorna et al., 1996], and collagen-induced arthritis [Cantorna et al., 1998], murine models of human multiple sclerosis and rheumatoid arthritis, respectively.

The focus of this review is on the role of vitamin D in insulin-dependent Diabetes mellitus (IDDM), also commonly referred to as type I, autoimmune, or juvenile-onset diabetes. The function of vitamin D as a genetic and environmental determining factor for IDDM, the protective role of $1,25(OH)_2D_3$ and its analogs in a mouse model of IDDM, and the possible mechanisms by which this protection occurs will be discussed.

VITAMIN D AS AN ENVIRONMENTAL AND GENETIC RISK FACTOR FOR IDDM

Unlike most non-infectious chronic diseases, the incidence of IDDM follows geographic patterns. In 1988, the results from the first international collaborative effort to assess geographic patterns on risk of IDDM were reported [Diabetes Epidemiology Research International Group, 1988]. This report was compiled from age- and sex-specific cases of

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IDDM from 1978–1980, representing approximately 50 million children younger than 15 years of age from 22 countries on four continents. The authors found a significant correlation between increased IDDM risk and lower average yearly temperatures which was strongly associated with latitudes increasing in distance from the equator. An increase in average monthly 25-hydroxyvitamin D_3 serum levels have been shown to correlate with higher average monthly temperatures and approximated monthly amounts of sunlight [Stryd et al., 1979]. Similar geographic variations have also been suggested for the prevalence of two other autoimmune diseases, multiple sclerosis and rheumatoid arthritis [reviewed in Cantorna, 2000]. Together, these results suggest that vitamin D may be an environmental determinant in the risk for IDDM.

The implication of vitamin D-deficiency as a risk factor for IDDM was more directly evidenced by three population studies investigating the association between early childhood vitamin D supplementation and incidence of IDDM. The first study, published in 1999, was designed to assess the correlation between vitamin D supplementation during the first year of life with development of IDDM by 15 years of age [The EURODIAB Substudy 2 Study Group, 1999]. This case-controlled study was based on a population of 820 diabetic patients and 2335 control subjects from seven centers in Europe. Although neither the dose of vitamin D supplementation nor measurement of serum vitamin D metabolite levels were taken into consideration, the authors reported that vitamin D supplementation during the first year of life is associated with a decreased risk of IDDM. The second study, published 1-year later, expanded upon the EURODIAB study by examining the association between IDDM onset and vitamin D supplements or cod liver oil taken by the mother during pregnancy or by the infant during the first year of life [Stene et al., 2000]. The results of this study were based on a survey of 85 diabetic and 1071 control subjects from a county in Norway. The authors reported a negative association between maternal intake of cod liver oil during pregnancy and onset of IDDM in their children by 15 years of age. However, the authors found no significant association between maternal intake of vitamin D (in the form of multivitamins) and IDDM risk. In contrast to the EURODIAB study, there was no significant protection from IDDM risk when infants were fed either cod liver oil or vitamin D supplements. As with the EURODIAB study, neither the dose of vitamin D nor the serum levels of its metabolites were recorded. The most recent and only prospective study of vitamin D intake and risk of IDDM was based on the assessment of vitamin D intake during infancy of 10,821 children in two regions of Northern Finland [Hypponen et al., 2001]. Based on information provided by each mother after her child's first year of life, both the frequency and dose of vitamin D supplements were recorded. In accordance with the dietary guidelines in Finland from 1964–1975, vitamin D supplementation was noted as below (<2000 IU), within (2000 IU), or above (>2000 IU) the recommended daily dose. The authors report that both the frequency and dose of vitamin D supplementation is associated with risk of IDDM by 30 years of age. Specifically, infants that received vitamin D supplements regularly, or those that received at minimum the recommended daily dose, had a significant reduction in developing IDDM by the age of 30 years than those infants that received less than 2000 IU/day. Albeit a high level of vitamin D was used as a standard in this study, the results provide a convincing argument for the use of vitamin D supplements in infancy as a preventative measure against IDDM [reviewed in Harris, 2002].

In addition to the environmental role of vitamin D on IDDM risk, certain allelic variations in the VDR may also be of genetic risk for IDDM. In humans, the gene encoding the VDR consists of 14 exons spanning a region of approximately 75 kb of genomic DNA on the 12q chromosome [Zmuda et al., 2000]. Four common allelic variants of the VDR gene have been identified and are described by the endonuclease restriction sites from which they are formed. *Fok*I, *Bsm*I, *Apa*I, and *Taq*I restriction site polymorphisms occur in exon 2, the intron between exons 8 and 9 (*Bsm*I and *Apa*I), and exon 9, respectively. Unlike loss of function mutations in the VDR which result in hereditary vitamin D-resistant rickets, allelic variants of the VDR gene translate into functional VDR protein. These variants have been extensively studied as markers for susceptibility to a variety of diseases including osteoporosis, cancer, hyperparathyroidism, and psoriasis.

Several reports now indicate that IDDM may be included among the list of diseases

genetically determined, at least in part, by VDR gene polymorphisms. Studies of these polymorphisms indicate that allelic variations in the VDR gene influence genetic susceptibility to IDDM in Indian Asian [McDermott et al., 1997], Taiwanese [Chang et al., 2000], German [Pani et al., 2000], and Japanese [Ban et al., 2001] populations. To summarize, subjects were genotyped for the presence of VDR polymorphisms using various combinations of the *Fok*I, *Bsm*I, *Apa*I, and *Taq*I restriction enzymes. These reports indicate that the *Bsm*I restriction site alleles of the VDR gene influence susceptibility in Indian Asian, Taiwanese, and German populations. Ban et al. [2001] studied the association between IDDM and the presence of VDR alleles containing only the *Fok*I restriction site. The authors found that, this VDR polymorphism, which contains an additional three amino acids at the N-terminus of the protein, influences genetic susceptibility to IDDM in the Japanese population. Together with the above described studies, these findings suggest that vitamin D may contribute to both the heterogeneous environmental determinants and polygenic factors that lead to IDDM.

PROTECTIVE ROLE OF VITAMIN D AND ITS ANALOGS IN IDDM: STUDIES IN THE NOD MOUSE

As is typical in the study of human diseases, the previously described reports of an association between vitamin D and human IDDM were limited to correlations from natural experiments. Despite the obvious species differences, animal models of human disease can afford greater manipulation and a more direct assessment of disease pathology. One such example is the non-obese diabetic mouse (NOD), a murine model of human IDDM. The NOD mouse strain was serendipitously developed over 20 years, during the production of a strain of mice that developed cataracts [reviewed in Tochino, 1987]. Similar to the human disease, NOD mice develop hyperglycemia as a result of a T-cell mediated autoimmune reaction against the insulin-producing β-cells of the islets of Langerhans in the pancreas. The prediabetic state is hallmarked both by circulating autoantibodies to proteins produced by the β-cell, and by infiltration of the islets by leukocytes, a condition known as insulitis. The loss of glycemic control results in polydipsia, polyuria, and excessive weight loss if not treated with exogenous insulin. The similar disease pathogenesis to human IDDM and the spontaneous nature of this disease course have made the NOD mouse an excellent tool to study the interplay between environmental factors and genetics in the pathogenesis of IDDM.

More than 125 therapies have been reported to suppress autoreactive T-cell function or to stimulate the immune system in the prevention of IDDM in the NOD mouse [Atkinson and Leiter, 1999]. Both of these immunomodulatory properties have been attributed to $1,25(OH)_2D_3$, the biologically active form of vitamin D. In a pilot study reported in 1992, Mathieu et al. [1992] examined the ability of $1,25(OH)_2D_3$ to inhibit insulitis in the NOD mouse. Insulitis, a necessary but insufficient determinant of IDDM onset, appears in the NOD mouse between 20 and 40 days of age, with 100% incidence by 200 days of age in both male and female mice. In this study, NOD mice were injected intraperitoneally (i.p.) with vehicle (Arachis oil) or 5 μg $1,25(OH)_2D_3$/kg body weight every other day from 21 to 99 days of age. Pancreata were fixed and stained for lymphocyte infiltration into the islets. The authors report that no mouse showed insulitis at 21 days of age, but that this condition appeared in 75% of the mice by 100 days of age in the vehicle-treated group. $1,25(OH)_2D_3$ offered partial protection against insulitis, shown by a reduction to 42% by 100 days of age. This treatment regimen of $1,25(OH)_2D_3$ resulted in a significant, yet reportedly tolerable, increase in serum calcium concentration, a typical side-effect of treating with pharmacological doses of $1,25(OH)_2D_3$.

The ability of $1,25(OH)_2D_3$ to reduce insulitis in NOD mice led to the examination of the hormone's role and its analogs in disease prevention. Both $1,25(OH)_2D_3$ and its non-hypercalcemic analog, $1,25(OH)_2$-20-epi-22-oxa-24,26,27,-trishomovitamin D (KH1060), have been shown to reduce IDDM onset in NOD mice [Mathieu et al., 1995]. Here, female NOD mice were dosed with 5 μg $1,25(OH)_2D_3$/kg, 0.2 μg KH1060/kg, 0.4 μg KH1060/kg or vehicle (Arachis oil) i.p. every other day from day 21. By 200 days, 55% of the control mice have developed diabetes. As shown in previous work [Mathieu et al., 1994], 0.5 μg $1,25(OH)_2D_3$/kg reduced the onset to 18%. Both the 0.2 μg/kg and 0.4 μg/kg doses of KH1060 were able to reduce diabetes onset to 22 and 11%, respectively. This

reduction in disease onset was paralleled by a reduction in insulitis in all three treatment groups. The authors reported normal serum calcium values for mice treated with either $1,25(OH)_2D_3$ or the low dose of KH1060, although $1,25(OH)_2D_3$ caused significant bone decalcification as determined by bone ash. Only the higher dose of KH1060 caused a significant increase in serum calcium concentration, although the bone calcium content remained normal with either dose of the analog.

Our laboratory is interested in the regulation of autoimmunity by vitamin D and its analogs, and we are also investigating the role of $1,25(OH)_2D_3$ and its analogs in IDDM using the NOD mouse model. Similar to the results published by Mathieu et al., we have seen that $1,25(OH)_2D_3$ does not offer complete protection against IDDM onset in NOD mice when administered i.p. every other day. However, our results show that all NOD mice are completely resistant to IDDM by 200 days of age when a daily dose of 50 ng $1,25(OH)_2D_3$ is administered orally through the diet from weaning (unpublished data). In addition, we have seen that both male and female vitamin D-deficient NOD mice display a significant earlier mean day of onset and greater overall incidence of diabetes by 200 days of age than their vitamin D-sufficient counterparts. As expected, mice treated with 50 ng $1,25(OH)_2D_3$/day are hypercalcemic as determined by serum calcium concentrations. In an attempt to reduce the hypercalcemia, we have orally administered lower amounts of 10 and 25 ng $1,25(OH)_2D_3$ to NOD mice, but found these to be ineffective in completely preventing the onset of IDDM. The differences between our results and those previously published may be explained in two ways. First, the mode of delivery of $1,25(OH)_2D_3$ may explain the differences in its ability to prevent IDDM in the NOD mouse. Since $1,25(OH)_2D_3$ is rapidly cleared from the body, continual consumption of smaller amounts may result in more consistent circulating levels of both the hormone and calcium than a single large dose of $1,25(OH)_2D_3$ every 48 h. This suggests that oral administration of $1,25(OH)_2D_3$, or preferably a non-hypercalcemic analog, would be more clinically relevant for the prevention of IDDM in humans. Second, Mathieu et al. did not induce hypercalemia with their injection method, whereas we observed marked increase in serum calcium levels in all NOD mice treated with $1,25(OH)_2D_3$, regardless of vitamin D status. This would argue that hypercalcemia may be a contributing factor to complete prevention of IDDM in NOD mice. The role of calcium in IDDM prevention in the NOD mouse is currently being investigated in our laboratory.

The studies described here have demonstrated that $1,25(OH)_2D_3$ and one of its analog is able to confer at least partial protection against IDDM onset in the NOD mouse if the treatment regimen is started before insulitis is detected. However, vitamin D analogs would prove more clinically applicable if they could halt the progression to overt diabetes in patients identified to be in a prediabetic state. Two analogs of $1,25(OH)_2D_3$ have been studied for their potential to prevent the progression to diabetes in NOD mice post-insulitis. A non-hypercalcemic analog of $1,25(OH)_2D_3$, MC1288 (20-epi-$1,25(OH)_2D_3$), alone or in combination with the immunosuppressive agent cyclosporin A (CyA), was evaluated for its potential to prevent progression to overt diabetes [Casteels et al., 1998]. Pancreata from 70 day old female NOD mice were biopsied and analyzed for the presence of insulitis (71–83% among treatment groups). At 85 days of age, the mice were injected i.p. with vehicle (Arachis oil), 7.5 mg CyA/kg/day through 105 days, 0.1 μg MC1288/kg/2 days through 200 days, or a combination of the CyA and MC1288 regimens. Neither the CyA nor the MC1288 treatments were able to significantly reduce diabetes onset by day 200 as compared to vehicle controls (74, 70, and 65% respectively). However, the combination of CyA and MC1288 was able to reduce diabetic onset to 35%. In a separate study, late intervention with another analog of $1,25(OH)_2D_3$, 1α,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor vitamin D_3 (Ro 26-2198), was assessed for its ability to inhibit IDDM onset in NOD mice [Gregori et al., 2002]. Eight weeks old female NOD mice were orally treated five times in a week with vehicle (miglyol 812), or with 0.3 μg/kg Ro 26-2198 through 12 or 16 weeks of age. The authors report that not only Ro 26-2198 is able to sustain a state of benign insulitis, but the analog also reduced diabetes onset by 38 weeks of age from 90% in the control group, to 50 and 16% in the groups treated for 12 and 16 weeks, respectively. This dose of Ro 26-2198 was the maximum level that did not produce hypercalcemia in these mice. Taken together, these studies suggest that vitamin D analogs, either

alone or in combination with other immunosuppressive agents, may have potential therapeutic applications in preventing the progression from a clinically determined prediabetic state to IDDM.

POSSIBLE MECHANISMS OF VITAMIN D ACTION IN IDDM

Much attention has been given to investigating both the immunological events that trigger self-destruction of pancreatic β-cells and to the mechanisms of target cell elimination. Several mechanisms of IDDM initiation have been proposed, including the release of self-reactive T cells into the periphery, positive selection of self-antigen-specific T cells, the loss of peripheral tolerance, and the loss of regulatory T cell activation [reviewed in Delovitch and Singh, 1997]. A layer of complexity is added when the roles of both environmental and genetic factors are taken into consideration. That such a wide range of immunomodulatory agents have been shown to suppress diabetes onset suggests that the disease pathogenesis is also extremely complex. Progression of IDDM has been shown to involve infiltration into pancreatic islet cells by several types of immune cells including dendritic cells, macrophages, B cells, and CD4 + and CD8 + T cells [reviewed in Benoist and Mathis, 1997]. The involvements of these different cell types in β-cell destruction are not mutually exclusive, although the exact interplay between them remains a subject of debate. However, a disruption in the balance of Th1/Th2 cells leading to a predominant expression of islet antigen-specific Th1 cells seems to be central in IDDM disease pathogenesis.

T helper 1 (Th1) and T helper 2 (Th2) cells are subsets of CD4 + T cells typically characterized by their cytokine profiles and the subsequent immune functions they modulate. Th1 cells produce interferon γ (IFNγ), tumor necrosis factor β (TNF β), and interleukin 2 (IL-2) which activate cell-mediated immune responses. Th2 cells typically produce IL-4, -5, -6, -9, -10 and -13, which are responsible for the activation of humoral immunity [reviewed in Rabinovitch, 1998]. During immune homeostasis, a balance between Th1 and Th2 cells is achieved by the inhibitory actions of either cell type's cytokines on the differentiation and effector functions of the opposing cell type. A popular method of classifying cytokines is based on their function, rather than their origin. Hence, type 1 cytokines include those produced by Th1 cells with the addition of IL-12, and typically enhance cellular immunity while diminishing the humoral immune response. Type 2 cytokines include those produced by Th2 cells, and preferentially repress cell-mediated immune responses while activating humoral immunity.

In the NOD mouse, insulitis leads to β-cell destruction when type 1 cytokines produced by the infiltrating leukocytes dominate over the production of type 2 cytokines [reviewed in Rabinovitch, 1998]. One way in which $1,25(OH)_2D_3$ and its analogs may correct this imbalance is by directly modifying T cell activating cells and/or the cytokines which they produce. Dendritic cells (DCs) are found in most tissues, and are potent stimulators of both B and T cells [Banchereau and Steinman, 1998]. Immature DCs, although unable to prime T cells, efficiently capture and process antigens. This antigenic stimulation initiates the maturation process of the DCs. Some of the distinguishing features of mature DCs include a high level of cell-surface MHC class II molecules and accessory signals for T cell activation, and their ability to synthesize high levels of IL-12. When T helper precursor (Th0) cells are exposed to mature DCs and the IL-12, that they produce, Th1 cells are preferentially formed over Th2 cells. However, when IL-4 is present in the microenvironment, the production of Th1 cells is suppressed in favor of the formation of Th2 cells.

It has been suggested that IL-12 dependent Th1 cells are the primary initiators of cell-mediated immunity. Th1 cells produce high levels of the type 1 cytokines, IFNγ and IL-2, which activate macrophages and cytotoxic T cells, respectively, which in turn can directly destroy the β-cells of the pancreas [Adorini, 2001]. Neutralization of endogenous IL-12 has been shown to ameliorate both insulitis and diabetes in NOD mice when an anti-IL-12 antibody was administered through 30, but not 15 weeks of age, suggesting that IL-12 may be an important regulator of effector cell development and activation in IDDM [Fujihira et al., 2000]. Therefore, since $1,25(OH)_2D_3$ has been shown to inhibit the production of IFNγ, IL-2, and IL-12 [Lemire, 1995], this hormone may be able to disrupt both the initiation and the progression of the Th1-mediated pathogenesis of IDDM.

In addition to its ability to inhibit the production of many type 1 cytokines, $1,25(OH)_2D_3$ and its analogs may also suppress the activation of Th1 cells by the direct modification of DCs. Piemonti et al. [2000] have shown that $1,25(OH)_2D_3$ is able to inhibit differentiation and maturation of cultured human monocyte-derived DCs into potent antigen presenting cells. More importantly, these DCs cultured in the presence of $1,25(OH)_2D_3$ were unable to effectively stimulate T cells in a mixed lymphocyte reaction. Further studies have shown that culturing of bone marrow cells with an analog of $1,25(OH)_2D_3$ resulted in a population of immature DCs unable to produce high levels of IL-12 [Griffin et al., 2001]. These DCs remained in an immature state and resisted further maturational stimuli, even after the analog was withdrawn from the culture medium. The authors also observed an increased number of mature DCs in the lymph nodes of VDR knock-out mice, suggesting a VDR-mediated pathway of $1,25(OH)_2D_3$ action on DC maturation, and hence their ability to activate Th1 cells in vivo.

$1,25(OH)_2D_3$ and its analogs may also protect against IDDM by enhancing the presence and/or function of suppressor T cells. Conventionally termed regulatory T (T_R) cells, this subset of CD4 + T cells has become widely recognized for its suppression of T-cell mediated immunity. T_R cells are predominantly present in a small subset of the naturally occurring CD4 + CD25 + T cell population derived from the thymus [Maloy and Powrie, 2001]. From their work with $1,25(OH)_2D_3$ and its analog, KH1060, on the repression of IDDM onset in the NOD mouse, Mathieu et al. [1994, 1995] suggested the presence of vitamin D-mediated restoration of suppressor T cell activity both in vitro and in vivo. A more recent study suggests that these regulatory T cells that control IDDM have the CD4 + CD25 + phenotype [Salomon et al., 2000]. In their study with the $1,25(OH)_2D_3$ analog, Ro 26-2198, Gregori et al. [2002] observed a significant increase in CD4 + CD25 + T cells in the pancreatic lymph nodes in analog-treated NOD mice. The authors suggest that this $1,25(OH)_2D_3$ analog-mediated increase in the percentage of CD4 + CD8 + T_R cells may be attributed to the formation of immature DCs with a tolerogenic phenotype that are able to induce CD4 + T cells with regulatory properties.

CONCLUSION

Over the past 20 years, the field of vitamin D and autoimmune diabetes has advanced from a potential association to conclusive evidence of the protective role of $1,25(OH)_2D_3$ and its analogs in IDDM. The possible mechanisms of vitamin D's action in IDDM disease pathogenesis are not limited to those discussed in this review, and others may have yet to be revealed. Regardless, we are confident that continued investigations into the therapeutic potential of vitamin D analogs will eventually lead to their clinical application in the prevention and treatment of IDDM and other autoimmune disorders.

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