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Vitamin D contributes to mast cell stabilization

Running title: VitD maintains mast cell homeostasis

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Abstract

Background and aims: Mast cells are the major effector cells in allergic disorders and many other informatory disorders. The mechanism of mast cell stabilization is not fully understood. Cumulative reports indicate that vitamin D (VitD) contributes to the homeostasis in the body. This study tests a hypothesis that VitD is required in the maintenance of the stability of mast cells.

Methods: The stability of mast cell lines, HMC1 cells, RBL-2H3 cells, p815 cells and mouse bone marrow-derived mast cells (BMMC) was tested in the presence or absence of VitD3.

Results: mast cells activated automatically in a vitamin D (VitD)-deficient environment. Exposure to calcitriol in the culture increased the expression of VitD receptor (VDR) in mast cells. VDR formed complexes with Lyn in mast cells to inhibit the binding of Lyn to the β chain of FcεRI and MyD88, which decreased the phosphorylation of Syk, decreased the levels of MAPK and NF-κB. VDR bound to the promoter of TNF-α to decrease the

acetylation of histone H3/H4, RNA polymerase II and OCT1 (a transcription factor of TNF-α) at the promoter locus and repressed the expression of TNF-α in mast cells.

Conclusions: The data demonstrate that VitD is required to maintain the stability of mast cells. The deficiency of VitD results in mast cell activation.

Keywords: Mast cell; Vitamin D; Calcitriol; Lyn tyrosine kinase; Syk tyrosine kinase.

Introduction

Mast cells are inflammatory effector cells, which are involved in the pathogenesis of a large number of diseases in the body, including allergic responses, autoimmunity, chronic inflammation and cancer (1). In allergic response, IgE antibodies bind to the high affinity IgE receptors to form complexes on the surface of mast cells to make mast cells sensitized. Upon re-exposure to specific antigens, the antigens bind to the complexes of IgE/FcεRI to activate mast cells. The mast cells then release proinflammatory mediators to initiate allergic responses (2). Besides, mast cells also can be activated by other factors, such as microbial products, temperature changes, express Toll like receptors (TLR). Thus, microbial products can activate mast cells (3, 4). Although the research about mast cells has advanced rapidly in the recent years, the inhibitory effects of various remedies on mast cell activation are temporary. Thus, to understand the inhibitory mechanism on mast cell activation is of significance.

In allergic environment, one of the important events in mast cell activation is the cross-linking of the high affinity IgE receptors, FcεRI, by specific antigens. The FcεRI is a tetrameric complex consisting of three sub units, including an IgE-binding α subunit, a tetraspan β subunit and the disulfide-linked γ subunits (5). It is reported that the FcεRIβ functions as a signal amplifier in mast cells and plays and important role in augmenting the allergic reaction-induced mast cell activation (6). The subsequent biochemical reactions include the Src family kinase Lyn binds to the β unit; followed by activation of spleen tyrosine kinase (Syk) and Syk phosphorylates linker for activation of T cells (LAT), the latter serves as a scaffold for forming the signaling complex that triggers degranulation and cytokine gene transcription (7). Although the mechanism about mast cell activation has been extensively studied, how to maintain the stability of mast cells is less clear.

Vitamin D (VitD) is involved in the suppression of a number of biochemical reactions (8). VitD can be endogenously synthesized in the skin upon irradiating by sunlight, or absorbed from food intake. The physiological role of VitD is to maintain the homeostasis of calcium and metabolism; only in recently its role in immune regulation has been recognized (9). VitD3 is converted to 25(OH)D_2 and further converted to calcitriol in the kidney or some immune cells (10). Calcitriol is the active form of VitD3 (11). By binding to gene promoters, VDR represses target gene transcriptions and inhibits the gene expressions (12). Mast cells express CYP27B1 that can convert 25(OH)D_2 to calcitriol to

inhibit IgE-mediated mast cell activation (13). Whether VitD can maintain the stability of mast cells is to be further investigated.

Based on the information above, we hypothesize that VitD contributes to the stabilization of mast cells. In the present study, we observed that mast cells activated automatically in a VitD deficient environment, which was abolished by the presence of VitD.

Materials and methods

Reagents

The shRNA kit of VDR, antibodies of VDR (D-6; sc13133), OCT1 (N-12), CYP27B1, FcεRI, Lyn, Syk, MAPK p38, NF-κB, acetylated histone H3/H4, RNA polymerase II and OCT1 were purchased from Santa Cruz Biotech (Santa Cruz, CA). The antibody of RMCP II was purchased from the Biomart (Beijing, China). The ELISA reagent kits of histamine and TNF-α were purchased from R&D Systems (Minneapolis, MN). The reagents for quantitative RT-PCR and Western blotting were purchased from Invitrogen (Carlsbad, CA). The ELISA kit of RMCP II was purchased from Biocampare (South San Francisco, CA). The biotinylated anti-c-kit antibody [2B8 (Biotin) (ab25022)] was purchased from Abcam (Cambridge, MA). The streptavidin magnetic beads were purchased from Miltenyi Biotech (San Diego, CA). The anti-DNP IgE antibody, calcitriol, reagents for immunoprecipitation and chromatin immunoprecipitation were purchased from Sigma Aldrich (St. Louis., MO). The endotoxin levels in all reagents were detected using the

Limulus assay (Limulus amebocyte lysate QCL 1000, Bio Whittaker, Walkersville, MD, USA). The reagents used in this study contained <0.2U endotoxin/10 μg reagents.

Animals

Male BALB/c mice (6-8 week old) and Sprague Dawley rats (about 200 g body weights) were purchased from the Guangzhou Experimental Animal Center (Guangzhou, China). The mice/rats were maintained in a specific pathogen-free environment with accessing food and water freely. The using mouse/rat in the present study was approved by the Animal Ethics Committee at Shenzhen University.

Treatment of mice with VitD-special diet

Grouped mice were fed with VitD-deficient diet, or VitD-sufficient diet, or VitD-supplement diet for one week. The mice were then used for further experiments. The VitD-special mouse food was provided by the Guangzhou Experimental Animal Center (Guangzhou, China).

Generation of BMMC

Following our established procedures (14), BMMCs were generated. More than 99% c-kit positive cells were detected in the generated BMMCs by flow cytometry.

Cell culture

The human mast cell line, HMC1 cells, rat mast cell line, RBL-2H3 cells (RBL, in short) and mouse mast cell lines, p815 cells, were purchased from ATCC (Manassas, VA, USA). The mast cells were cultured with RPMI1640 medium supplemented with 10% fetal bovine serum, 0.1 mg/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine with or without the addition of calcitriol (10 nM). The medium was changed in 2-3 days. The cell viability was greater than 99% before using in further experiments as checked by Trypan exclusive assay.

Enzyme-linked immunosorbent assay (ELISA)

The levels of histamine and TNF-α in the culture supernatant were determined by ELISA with commercial reagent kits following the manufacturer's instructions.

Sensitizing BMMCs and RBL cells with IgE

BMMC and RBL cells were cultured in the presence of DNP specific IgE (200 ng/ml) overnight. The cells were washed with fresh warm medium and cultured with or without calcitriol (10 nM) and challenged with DNP. Two hours after the addition of DNP, the supernatant was collected and analyzed by ELISA to determine the levels of histamine and TNF-α.

Assessment of mast cell activation

The levels of histamine and TNF-α in the culture supernatant or the sera were determined with ELISA with commercial reagent kits following the manufacturer's instructions. The results were regarded as the indicators of mast cell activation.

Real time quantitative RT-PCR (RT-qPCR)

The mast cells were harvested after experiment. Total RNA was extracted from the mast cells with TRIzol reagents. The cDNA was synthesized with the RNA using a reverse transcription reagent kit following the manufacturer's instructions. The samples were subjected to qPCR in a qPCR device (MiniOpticon, Bio-Rad) with the SYBR Green Master Mix. Primers used in the experiments are listed in Table 1. The results were calculated with the $2^{-\Delta\Delta Ct}$ and presented as fold change against control groups.

Western blotting

The total protein was extracted from the mast cells. The protein was fractioned by SDS-PAGE and transferred onto a PVDF membrane. The membrane was treated with 5% skim milk for half an hour to block non-specific binding and incubated with the first antibodies of interest or isotype IgG overnight at 4 °C, followed by incubating with the second antibodies (conjugated with peroxidase) for 1 h at room temperature. The membrane was washed 3 times with Tris-buffered saline after each time of incubation. The immune blots on the membrane were developed with the enhanced

chemiluminescence. The results were photographed with an imaging device (UVI, Cambridge, UK).

Immunoprecipitation (IP)

Mast cells were collected after related experiments. Cellular extracts were prepared with the mast cells and precleared by incubating with protein G agarose beads for 1 h at 4 C. The supernatant was collected by centrifugation and incubated with the antibodies of interest or isotype IgG overnight at 4 °C. The immune complexes were precipitated by protein G agarose beads at 4 C for 1 h. The beads were collected by centrifugation. The proteins on the beads were eluted with eluting buffer and analyzed by Western blotting.

Chromatin IP (ChIP)

Mast cells were harvested after related experiments and fixed with 1% formalin for 15 min to cross link DNA to the bound proteins. The cells were sonicated to shear the DNA into small pieces (200-500 bp). The samples were precleared, incubated with antibodies and precipitated as described in IP procedures above. The beads were collected and treated with eluting buffer. The DNA was recovered from the samples by reverse cross linking at 65 °C for 4 h; the proteins were removed by digesting with proteinase K at 45 °C for 1 h, then DNA was precipitated by phenol/chloroform extraction and ethanol precipitation. The DNA was analyzed by qPCR on a qPCR device. The primers of TNF-α promoter used in the present study include agactgaaggtttagggccc and

gctggctgagtgtgaaacaa (-200 to -956). The results were presented as fold change against the input (the DNA samples before adding antibodies).

Development of a food allergy mouse or rat model

BALB/c mice or Sprague Dawley rats were fed with ovalbumin (OVA; 0.1 mg/mouse, 1 mg/rat) mixing with cholera toxin (0.02 mg/mouse; 0.1 mg/rat) in 0.3 ml saline weekly for 4 weeks. The mice were used as the "sensitized mice" or “sensitized rats” in related experiments.

Purification of rat peritoneal mast cells

Following published procedures (15), rat peritoneal mast cells were purified. Briefly, the rats were under general anesthesia, the peritoneal cavity was irrigated with 20 ml HEPES Tyrode's buffer [10 mM HEPES, 5 mM KCl, 136 mM NaCl, 2 mM $CaCl_2$, 2.75 mM $MgCl_2$, 5.6 mM glucose, 0.6 mM NaH_2PO_4, 11 mM $NaHCO_3$, and 1% bovine serum albumin (BSA), pH 7.4]. The irrigating fluids containing mast cells were recovered 5 min later. The cells were treated by gradient density centrifugation (190 × g) for 10 min at 4 °C. The cells were collected and stained with biotinylated anti-rat c-kit (1:500) for 1 h at 4 °C, washed with PBS, incubated with streptavidin-magnetic beads for 1 h at 4 °C. The cells were filtered by passing through a column in a magnetic field. The purity of the mast cells was greater than 98% as assessed by flow cytometry labeled with the anti-RMCP-II antibody. Briefly, the cells were fixed with 1% paraformaldehyde/0.1 Tritoon-100 for 2 h and incubated with the mouse anti-rat RMCP II antibody (1:300) for 1 h at

4 °C. After washing, the cells were incubated with rabbit anti-mouse IgG (labeled with FITC) for 1 h at 4°C. The cells were washed again and analyzed with a flow cytometer (FACSCanto II, BD Bioscience).

Statistics

The data are presented as mean ± SD. The difference between two groups was determined by Student t test or ANOVA followed by the Bonferroni corrections if more than two groups. $P<0.05$ was set as the significant criterion.

Results

Mast cells activate automatically in a VitD-deficient environment

We cultured BMMC, HMC1, RBL-2H3 (RBL, in short) and p815 cells in RPMI1640 medium with or without the presence of calcitriol for 3 days. The supernatant was collected and analyzed by ELISA. The results showed that about 15-30% histamine and TNF-α were released to the culture medium from the mast cells without the presence of calcitriol (the active form of VitD3), which was inhibited by the presence of calcitriol (Fig. 1A-B). The results indicate that, in a VitD deficient environment, mast cells activate automatically, which can be inhibited by the presence of calcitriol. In addition, the cell viability of BMMC, HMC1, RBL and p815 cells was 98.4%, 98.8%, 99.1% and 98.2%, respectively as observed at the end of the culture. The results demonstrate that the mediators were released from live mast cells.

We next treated naive mice and sensitized mice with VitD deficient diet or VitD supplemented diet for one week. The blood samples were collected at the sacrifice. The levels of histamine and TNF-α in the sera were determined by ELISA. The results showed that the levels of histamine and TNF-α in the sera were significantly higher in both naive mice and sensitized mice fed with VitD deficient diet as compared to the mice fed with VitD supplemented diet (Fig. 1C-D).

VitD inhibits the antigen/IgE response-induced mast cell activation

To clarify if VitD also inhibits the antigen/IgE response-induced mast cell activation, we sensitized RBL cells and BMMC with DNP-specific IgE. The mast cells were challenged with DNP in the culture with or without the presence of calcitriol. The results showed that the challenge with antigen markedly induced histamine and TNF-α release, which was abolished by the presence of calcitriol (Fig. 2A-B). The results indicate that VitD can non-specifically attenuate the antigen/antibody response-induced mast cell activation.

On the other hand, we fed sensitized mice with VitD deficient diet or VitD sufficient diet or VitD supplemented diet for 7 days. The mice were challenged with the specific antigen, OVA, via gavage-feeding on day 7 and sacrificed 4 h later. The blood samples were collected and analyzed by ELISA. The results showed that the indicators of mast cell activation, the levels of histamine and TNF-α, were higher in mice fed with VitD deficient and VitD sufficient diet as compared to mice fed with VitD supplemented diet (Fig. 2C-D).

Calcitriol increases VDR expression in mast cells

Next we assessed the role of VitD in the regulation of VDR expression in mast cells. After exposure to calcitriol in the culture for 3 days, BMMC, HMC1 cells, RBL cells and p815 cells were collected and analyzed by RT-qPCR and Western blotting. The results showed that exposure to calcitriol markedly increased the expression of VDR in the mast cells (Fig. 3A-B).

On the other hand, we fed sensitized rats with VitD-deficient, or VitD-sufficient, or VitD-supplement, diet for 7 days. The peritoneal mast cells were collected and purified (Fig. 3C) and analyzed for VDR expression. The results showed that the expression of VDR of mast cells in sensitized rats fed with VitD-deficient or VitD-sufficient diet showed lower VDR expression than that in naïve rats, while mast cells from those fed with VitD-supplement diet showed higher levels of VDR (Fig. 3D-E).

VDR binds to Lyn to interrupt the signal transduction pathway of mast cell activation

We hypothesize that mast cells activate automatically without the presence of VDR. It is reported that VDR can interact with other molecules in the cytoplasm to modulate their functional status (16). We wondered if VDR interacted with the components of mast cell activation signal transduction pathways. To test this, we observed the role of VDR in the well-established IgE/FcεRI pathway (6) and LPS/TLR4/MyD88 pathway (17). We performed immunoprecipitation with mast cell extracts. The results showed that VDR

bound to the Lyn to form a complex (Fig. 4A), which resulted in inhibiting the binding of Lyn to the β chain of FcεRI (Fig. 4B) and to the MyD88 (Fig. 4C, inhibiting the Syk phosphorylation (Fig. 4D), and blocked the downstream signal transduction pathway, including mitogen-activated protein kinase (MAPK) p38 and NF-κB (Fig. 4F-H).

VDR binds to the promoters of TNF-α to inhibit gene transcription of TNF-α in mast cells

The data reported above indicate that VitD contributes to mast cell stability to prevent mast cell activation. VDR can bind to gene transcription apparatus to regulate gene expression. We inferred that VDR might bind to those molecules related to mast cell degranulation to regulate their functions. To test this, after exposing mast cells to calcitriol or cultured in medium alone in the culture for 3 days, we prepared cell extracts with the mast cells. The extracts were analyzed by chromatin immunoprecipitation. The results showed that VDR was detected at the promoter loci of TNF-α in mast cells cultured in the presence of calcitriol, while the levels of VDR were much less at the TNF-α promoter loci of mast cells cultured in the absence of calcitriol (Fig. 5A). We also observed that the presence of calcitriol resulted in less levels of acetylated H3 and H4, RNA polymerase II and OCT1 (OCT-1 binds to the TNF-α promoter to initiate TNF-α expression (18)), the transcription factors of TNF-α, at the promoter locus, which were significantly higher in the mast cells cultured in medium alone (Fig. B-E). The results indicate that VitD deficiency can result in higher gene transcription activities of TNF-α,

which can be prevented in the presence of calcitriol in the culture. The data were corroborated by the assessment of TNF-α mRNA in the mast cells (Fig. 5F).

Discussion

Mast cell activation triggers mast cell degranulation to release proinflammatory mediators, which is associated with the pathogenesis of a large number of informatory disorders (1). Although many mast cell stabilizers and mast cell mediator antagonists have been designed, produced and have good inhibitory effects on mast cell activation, the effects only last temporarily (19). The present study revealed a mechanistic phenomenon that mast cells required the presence of VitD to maintain the stability. Mast cells released mediators automatically in a VitD deficient environment without the presence of any known activators. The presence of VitD inhibited the IgE-sensitized mast cell activation. The data demonstrate that VitD plays a crucial role in the maintenance of the stabilization of mast cells in both quiescent condition and sensitization.

This study revealed a previous unknown phenomenon that VDR bound to the Lyn to prevent Lyn to bind to the β chain of FcεRI and MyD88, the two major pathways in the mast cell activation. Lyn is the first component to be activated immediately after the FcεRI crosslinking by multivalent antigens or exposure to microbial product LPS, during which Lyn binds to the β chain of FcεRI (6) or MyD88 (17). Blocking Lyn can block the FcεRI activation or LPS-induced signal propagation and inhibit mast cell activation (17,

20). We also observed that the VDR binding to the β chain of FcεRI prevented the Syk phosphorylation. Syk is another kinase that can be activated by Lyn. The activation of Syk further activates LAT; the latter is involved in triggering mast cell degranulation (21). Since the expression of VDR is maintained by sufficient VitD, the insufficient or deficient VitD can be one of the causal factors of the deficiency/insufficiency of VDR in mast cells. The reasoning is supported by the present data. Others also found that VitD deficiency was correlated with the deficiency of VDR (22).

We selected two parameters, the histamine and TNF-α, as indicators of mast cell activation in the present study. Both mediators are pre-synthesized and deposited in mast cells, and can be released immediately upon activation. In the IgE-induced mast cell activation, to inhibit Sky and LAT can suppress the release of TNF-α and inhibit allergic response (23). Our data show that VDR not only interacts with the β chain of FcεRI in mast cells, but also binds to the promoter of TNF-α and represses its transcription and expression. The data indicate that VDR is involved in the biochemical reactions in both the cytoplasm and nuclei. Others also reported that VDR was involved in various acetyl-CoA-dependent biosynthetic pathways in tricarboxylic acid cycle in the cytoplasm (16). Mast cells produce a number of proinflammatory mediators, whether the expression of other mediators is also regulated by VitD is to be further investigated.

In summary, the present data show that VitD plays a critical role in the maintenance of the stabilization of mast cells. Mast cells activate automatically in the VitD deficient

environment. The data suggest that it is necessary to take the VitD metabolism abnormality into account when set the therapeutic strategy for mast cell-related diseases.

Conflict of interest: None to declare.

Author contributions: ZQL, XXL, SQQ, YY, MGL, LTY, LJL and SW performed experiments, analyzed data and reviewed the manuscript. PYZ, ZGL and PCY organized the study and supervised experiments. PCY designed the project and wrote the manuscript.

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Figure legends

Figure 1. Assessment of histamine and TNF-α. A-B, the levels of histamine (A) and TNF-α (B) in the culture supernatant of mast cell lines after culture in the presence of calcitriol (10 nM) or saline for 72 h. The data of A-B were summarized from 3 independent experiments. C-D, the serum levels of histamine (C) and TNF-α (D) in sensitized mice fed with VitD-deficient diet or

VitD-supplement diet. Each group consists of 6 mice. The bars are presented as mean ± SD. *p<0.01.

Figure 2. Assessment of effects of VitD on inhibiting mast cell activation. A-B, the levels of histamine (A) and TNF-α (B) in mast cell culture supernatant. The mast cells were sensitized by DNP-specific IgE and challenged by DNP in the presence of saline or calcitriol (10 nM) in the culture. C-D, the serum levels of histamine and TNF-α of mice. The mice were sensitized to OVA and challenged with OVA via gavage-feeding together with the treatment as denoted on the X axis. The data are presented as mean ± SD. Data of A and B were summarized from 3 independent experiments. In C and D, each group consists of 6 mice. *p<0.01.

Figure 3. VitD increases VDR in mast cells. A-B, the bars indicate the mRNA levels (A) and the immune blots indicate the protein levels (B) of VDR in BMMC, HMC1 cells, RBL cells and p815 cells after exposure to calcitriol (10 nM) in the culture for 3 days. C-E, sensitized rats were fed with the indicated diets (denoted on the X axis of D) for 7 days. The peritoneal mast cells were collected and purified. C, the gated histograms indicate the RMCP II staining in the purified mast cells. D-E, the levels of VDR mRNA (D) and protein (E) in the purified mast cells. The data of bars are presented as mean ± SD. *p<0.01. The experiments were repeated 3 times. The reactivity of the anti-VDR antibody for Western blotting includes human, rat and mouse (Santa Cruz Biotech; clone D-6; sc13133).

Figure 4. Assessment of the role of VDR in the signal transduction pathways of mast cells. Wild type BMMC and VDR-deficient BMMC were cultured in the presence or absence of calcitriol for 3 days. The cell extracts were prepared and analyzed by immunoprecipitation and Western

blotting. The immune blots indicate VDR binds to Lyn to form a complex (A), FcεRIβ/Lyn complex (B), MyD88/Lyn complex (C), Syk phosphorylation (D), VDR RNAi results (E), MAPK p38 (F), NF-κBp50 (G and NF-κBp65 (H). The data are from one of the 3 independent experiments.

Figure 5. VDR mediates calcitriol-induced TNF-α gene chromatin remolding in mast cells. Wild type BMMC and VDR-deficient BMMC were cultured in the presence or absence of calcitriol for 3 days. The cell extracts were prepared and analyzed by ChIP. The bars indicate the levels of VDR (A), acetylated H3/H4 (B-C), RNA polymerase II (Pol II, D), OCT1 (E; the TNF-α transcription factor) at the TNF-α promoter locus of BMMC. F, the bars indicate the TNF-α mRNA levels in BMMC. The data were summarized from 3 independent experiments. *p<0.01.

Table 1. Primers used in the present study

Molecules	Species	Forward	Reverse
VDR	Human	gccatccacaattccaggtc	tcccacccgatatcaccttg
VDR	Mouse	tatgacctgtgaaggctgca	ctgcacctcctcatctgtga
VDR	Rat	tcaccgatgtctccaaagct	gtctgcagcgtgttggatag
TNF-α promoter	Mouse	accctcacactcacaaacca	ggcagagaggaggttgactt