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Expression of retinoic acid receptors in intestinal mucosa and the effect of vitamin A on mucosal immunity

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ABSTRACT

Objective: To explore the mechanism of vitamin A (VA) modulation of mucosal immunity, the expression of retinoic acid receptors in intestinal mucosa was measured, and the effect of VA on intestinal dendritic cells (DCs) and mucosal cytokine production was examined.

Methods: The expression of retinoic acid receptor (RAR-α, RAR-β, RAR-γ, RXR-α, RXR-β, RXR-γ) mRNA, the distribution and number of DCs, and the protein secretion of interleukin (IL)-12, T-helper type 1/2 cells (interferon [IFN]-γ/IL-4), and regulatory (IL-10) cytokines in the mucosa of terminal ileum in normal rats and rats with VA deficiency (VAD) were detected by reverse transcriptase polymerase chain reaction, immunohistochemistry, and enzyme-linked immunosorbent assay, respectively. The effect of all-trans retinoic acid on the number and maturation of DCs and the gene expression of RAR-α and cytokines listed earlier in cultured Peyer's patches were examined by flow cytometry and reverse transcriptase polymerase chain reaction, respectively.

Results: In the intestinal mucosa of VAD rats, RAR-α mRNA was downregulated, DC number increased, the protein secretion of IL-12 was increased, but the secretion of IFN-γ and IL-10 decreased. In in vitro cultured Peyer's patches, all-trans retinoic acid promoted DC maturation, upregulated RAR-α mRNA, reduced IL-12 and IFN-γ, but increased IL-10 gene expression; these effects of all-trans retinoic acid were reversed when cultured with Ro 41-5253 (a specific antagonist of RAR-α).

Conclusion: Vitamin A may be potent in reducing intestinal inflammation and restoring impaired antibody responses in a VAD situation. The effect of VA on DCs could be an important mechanism contributing to altered mucosal immunity. RAR-α may mostly play a role in the action of VA.

Introduction

Many recent investigations have indicated that vitamin A (VA) deficiency (VAD) in children is still a serious public health issue in low-income countries [1]. Children with VAD have an increased incidence of respiratory tract and enteric infections, such as measles, pneumonia, diarrhea, etc. [2], but supplementation with VA has been shown to prevent these diseases and aid treatment [3]. Because VA is one of the most important microelements for childhood development, the positive effect of VA on mucosal immunity against infections has been widely accepted. Although VA has been shown to increase the production of secretory immunoglobulin (Ig) A in mucosa [4] and modulate the function of T and B lymphocytes [5], the mechanism for VA promotion of mucosal immunity remains unclear.

Retinoic acid receptors can be divided into RARs (α, β, γ) and RXRs (α, β, γ), whose distributions are tissue specific [6]. Retinoic acid (RA) exerts its effects on cellular growth, differentiation, and function exclusively by these nuclear receptors [7]. Our laboratory previously investigated the expression of these receptors in lymph nodes and thymus [8,9] and found that RAR-α plays a key role in the modulation of T and B lymphocytes by RA. In the present study, the expression of these retinoic acid receptors was examined for the first time in the intestinal mucosa of normal rats and rats with VAD to assess which receptor's expression is influenced most by VAD. Furthermore, in in vitro culture we used Ro 41-5253, which is a specific antagonist of RAR-α, to substantiate the role of RAR-α in RA modulation of intestinal mucosal immunity.

Dendritic cells (DCs), which are the most powerful antigen-presenting cells, are critical players in intestinal immune defense. Immature DCs reside dispersedly in the entire intestinal mucosa with prominent localization in the subepithelial dome of

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Peyer's patches (PPs), which are enriched in terminal ileum. There, DCs guard the sites of pathogen entry into the host, capture invading micro-organisms, and then migrate to interfollicular traffic areas of the PPs, gradually maturing during migration. Mature DCs initiate a primary immune response through expression of major histocompatibility complex and costimulatory molecules. Moreover, the interaction of DCs with T cells is important in directing T-helper type 0 (Th0) cell differentiation and in determining the resulting cytokine production profile and function [10]. The production of interleukin (IL)-12 (secreted mainly by DCs) and interferon-γ (IFN-γ; a Th1 cytokine) mediate Th1 differentiation, whereas the production of IL-4, IL-5, and IL-6 (Th2 cytokines) favors Th2 differentiation. In addition, IL-10 had been shown to skew T-cell responses toward T regulatory (Treg) cells that produce high levels of IL-10 and inhibit antigen-specific T-cell responses [11].

Previous studies from our laboratory [12] and other laboratories [13] have demonstrated that DCs are also the target cells of VA. Determining the role of VA in the differentiation, maturation, and function of intestinal DCs is therefore of great interest, because it could help to clarify the mechanism by which VA modulates intestinal mucosal immunity from the primary step of immune response. Rats with VAD were established as a model for in vivo studies, and PPs from the rats were cultured with all-trans RA (at-RA) and/or Ro 41-5253 in vitro to study the impact of VAD and VA supplementation on DCs and local cytokine production and on the retinoic acid receptors involved.

Materials and methods

Rat model of VAD

The methods used for establishing the rat model of VAD have been described elsewhere [14]. Maternal Sprague-Dawley rats (Animal Center of Shanghai Medical Collage, Fudan University) that had been pregnant for 10 d were fed a VA-deficient (TD.86143) diet (Harlan Teklad, Madison WI, USA) until weaning. Then the male weanling offspring were fed the same diet until the state of VAD was reached, and they were then sacrificed. All animals appeared to be healthy except for VAD-related characteristics. Animals in the control group were fed a VA-replete (TD.86143 + retinyl esters, 1200–3600 RE/kg) diet during the same period. The experimental work was approved by the local ethics committee for animal research.

PPs cultured in vitro

Male adult Sprague-Dawley rats in good nutritional condition were purchased from the same source. The terminal ileum was dissected, the serosa and muscle layers were carefully stripped, and after washing the tissue in Hanks solution free calcium and magnesium ions, the PPs were removed using curved scissors. The PPs were thereafter cut into 3- × 3-mm segments and placed in six-hole culture dishes with RPMI-1640 (GIBCO, Tulsa, OK, USA) supplemented with 10% heat-inactivated neonatal calf serum (Sijiqing, Hangzhou, PR China), 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37 °C in an environment of 5% CO_2. The experiments were organized into three groups: a control group, in which PPs were cultured in medium alone; an RA group, in which 1×10^{-6} mol/L of at-RA (Sigma, St. Louis, MO, USA) was added to the medium; and an RA + RO group, in which 1×10^{-6} mol/L of at-RA and 1×10^{-5} mol/L of Ro 41-5253 (kindly provided by F. Hoffmann-La Roche, Basel, Switzerland) was added to the medium. The segments were harvested 24 and 48 h later and disrupted mechanically with a 200 mesh stainless steel sieve [15] to prepare single-cell suspensions.

Immunohistochemistry

To determine the effect of VAD on intestinal DCs, the number of DCs in PPs of the terminal ileum was detected by OX-62–positive cells/mm^2 of tissue [16]. For OX-62 immunohistochemical staining, paraffin sections were incubated with mouse monoclonal anti–OX-62 (MCA1029 G, diluted 1/25; Serotec, Oxford, UK). After the unbound primary antibodies were removed, sections were incubated with horseradish peroxidase–conjugated secondary antibodies: sheep anti-mouse IgG (diluted 1/40; KPL, Gaithersburg, MD, USA). Immunoreactivity was visualized with 0.05% diaminobenzidine in Tris-HCl buffer (0.1 M, pH 7.6) containing 0.03% H_2O_2. The image analysis system used was Leica QWin (Leica, Cambridge, UK).

Flow cytometric analysis

The number and maturation of DCs in cultured PPs were evaluated by flow cytometry (FACSCalibur, BD, Franklin Lakes, NJ, USA) using the following fluorescence-labeled antibodies: phycoerythrin-conjugated anti–OX-62 (a specific surface marker for rat DC) and fluorescein isothiocyanate–conjugated anti–OX-6 (major histocompatibility complex class II) or fluorescein isothiocyanate–conjugated anti-CD86 (costimulatory molecule; all from Serotec). Corresponding isotype-matched monoclonal antibodies were used as negative controls. Results are expressed as the percentage of OX-62–positive cells and as fluorescence intensity of OX-6 and CD86.

RNA isolation and reverse transcription

To investigate the mRNA levels of retinoic acid receptors (RAR-α, β, γ; RXR-α, β, γ) in mucosa of normal rats and rats with VAD, mucosa of the terminal ileum was scraped and then put in a mortar and ground quickly with liquid nitrogen. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For the gene expression of RAR-α and cytokines (IL-12, IFN-γ, IL-4, and IL-10) in cultured PPs, total RNA was isolated from the prepared single-cell suspensions by TRIzol in the same way. The RNA concentration was measured by absorbance at 260 nm in a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). Two micrograms of total RNA was then reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega, Madison, WI, USA) according to the manufacturer's instructions. After reverse transcription, the 20-μL reaction was diluted to 100 μL so that 5 μL of RT product added to a polymerase chain reaction (PCR) mixture would be equivalent to the reverse transcription of 100 ng of total RNA.

Real-time quantitative PCR

Eva Green dye and realplex^4 (Eppendorf, Hamburg, Germany) were used for real-time PCR. β-Actin was used as a control for quality. The primer sequences, annealing temperatures, and PCR product sizes are listed in Table 1. Quantitative PCR was carried out in a 50-μL final volume: 3 mM $MgCl_2$, 0.2 μM of each primer, 5 μL of cDNA, and 2.5 U of Taq Gold DNA polymerase (SABC, Shanghai, PR China). The PCR conditions were as follows: 95 °C for the first 20 min, then 95 °C for 30 s, 54–60 °C (Table 1) for 30 s, and 72 °C for 40 s, for 40 cycles. Amplification specificity was checked using a melting curve according to the manufacturer's instructions. The relative number of molecules of each transcript was determined by interpolating the threshold cycle values of the unknown samples to each standard curve.

Enzyme-linked immunosorbent assay

To investigate the protein secretion of cytokines (IL-12, IFN-γ, IL-4, and IL-10) in mucosa of normal rats and rats with VAD, mucosa of the terminal ileum was scraped, weighed, and ground with liquid nitrogen. Every 50 mg of tissue was mixed with 1 mL of phosphate buffered saline, and after centrifugation at 17 500 × *g* for 15 min at 4 °C, the supernatants were harvested. Cytokine levels in the supernatants were measured with enzyme-linked immunosorbent assay kits (R&D, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analyses

Data are expressed as means ± standard deviations. For in vivo experiments, statistical significance was determined using a two independent-samples *t* test. For in vitro experiments, double factor analysis of variance was applied to examine group differences. To further determine which groups were different from the others, we conducted multiple comparisons with the Student-Newman-Keuls test. $P < 0.05$ was accepted as statistically significant.

Results

The gene expression of RAR-α, β, γ and RXR-α, β, γ in the mucosa of terminal ileum in normal rats and rats with VAD was measured by RT fluorescence quantitative PCR. The results showed that the RAR-α mRNA in VAD rats was significantly lower than in control rats ($n = 10$, $P = 0.029$). Few significant differences were seen in the rest of the receptors between VAD and control rats (Fig. 1).