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Iodine excess did not affect the global DNA methylation status and DNA methyltransferase expression in T and B lymphocytes from NOD.H-2^{h4} and Kunming mice

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ABSTRACT

Dysregulated DNA methylation in lymphocytes has been linked to various autoimmune disorders. Excessive iodine intake leads to lymphocyte dysfunction and contributes to autoimmune thyroiditis (AIT) flares in humans and animals. However, whether excessive iodine modifies the DNA methylation status in lymphocytes is unknown. Twenty NOD.H-2^{h4} mice and 20 Kunming mice were randomly divided into high iodine and control groups. We scored lymphatic infiltration in the thyroid by hematoxylin and eosin (H&E) staining and assayed serum thyroglobulin antibody (TgAb) levels by an indirect enzyme-linked immunosorbent assay. $CD3^+$ T cells and $CD19^+$ B cells were separated by flow cytometry. Global DNA methylation levels were examined by absorptiometry. Methylation of long interspersed nucleotide element-1 (LINE-1) repeats was detected with bisulfite sequencing PCR. Expression of DNA methyltransferase (DNMT) 1, DNMT3a and DNMT3b mRNA and protein were determined by real-time PCR and Western blot, respectively. We observed evident thyroiditis in the high-iodine-treated NOD.H-2^{h4} mice, while mice in the other three groups did not develop thyroiditis. No differences were found in the global methylation levels and methylation status of LINE-1 repeats in T and B lymphocytes from high-iodine-treated NOD.H-2^{h4} mice and Kunming mice compared with those from normal-iodine-supplemented controls. We did not find obvious changes in DNMT mRNA and protein expression levels in T and B lymphocytes among the studied groups. In conclusion, we showed for the first time that excess iodine did not affect the global methylation status or DNMT expression in T and B lymphocytes in NOD.H-2^{h4} and Kunming mice.

1. Introduction

Autoimmune thyroiditis (AIT) is a common, organ-specific autoimmune disorder characterized by lymphatic infiltration in the thyroid gland and autoantibodies in the serum [1]. Disruption of immune tolerance by imbalanced and dysfunctional T and B lymphocytes is an important immunological pathogenesis mechanism of AIT [2]. AIT develops and progresses when genetically predisposed individuals encounter certain environmental factors [3]. Iodine is an essential element for thyroid hormone synthesis; however, studies by our group and others have indicated that excess iodine is closely related to AIT onset in humans and animals [4,5]. Of the mechanisms that have been proposed for AIT development [6], high-iodine-induced oxidative stress injury is the most recognized and comprehensive [7] but is not yet fully understood.

Environmental factors affect gene expression through epigenetic modification [8]. DNA methylation is the most studied epigenetic modification pattern, occurring at cytosine residues mainly in the context of CpG dinucleotides, and is generally associated with transcriptional silencing [9]. DNA methylation is mediated and maintained by DNA methyltransferases (DNMTs). In particular, DNMT3a and DNMT3b are responsible for de novo methylation [10], while DNMT1 is required for methylation maintenance [11]. Changes in genome-wide DNA methylation may affect repetitive DNA sequences that are comparatively rich in CpG dinucleotides, such as long interspersed nucleotide element-1 (LINE-1) repeats [12]. In addition, the methylation

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status of LINE-1 has been reported to be a good indicator of the cellular 5-methylcytosine (5-mC) level [13]. DNA methylation plays an essential role in lymphocyte differentiation and functional regulation [14]. Aberrant DNA methylation patterns or DNMT expression in T and B lymphocytes could contribute to the development and progression of various autoimmune disorders [14,15], such as systemic lupus erythematosus (SLE) [16], type 1 diabetes mellitus (T1D) [17], and rheumatoid arthritis (RA) [18]. In recent years, a growing body of evidence has indicated that dysregulated DNA methylation and single nucleotide polymorphisms (SNPs) in DNMT genes play important roles in the etiology of autoimmune thyroid disease (AITD) [19–21]. As a ubiquitous epigenetic modification pattern, DNA methylation is reported to be affected by oxidative stress [22,23]. Oxidants decrease DNMT1 expression and induce DNA demethylation in T cells by inhibiting the extracellular-signal-regulated kinase pathway [24]. Adoptive transfer of oxidant-treated $CD4^+$ T cells into syngeneic animal recipients resulted in anti-dsDNA antibody production and glomerulonephritis [25].

Excessive iodine intake is closely associated with the immune dysfunction of lymphocytes in humans and animals [26,27]. However, whether excess iodine induces immune dysfunction and contributes to AIT flares by affecting the global DNA methylation status in lymphocytes is unknown. Therefore, the aim of this study was to observe whether excessive iodine modifies the global DNA methylation status in lymphocytes and if so, whether the modified global DNA methylation status participates in AIT pathogenesis. We used high-iodine-supplemented NOD.H-2^{h4} mice to establish a model of experimental autoimmune thyroiditis (EAT), which is considered a typical animal model for AIT in humans [28]. In addition, to explore the role of excess iodine independent of an autoimmune-prone genetic background, we also used Kunming mice with a normal genetic background. We demonstrated that excess iodine did not affect global DNA methylation level and DNMT expression in T and B lymphocytes in either NOD.H-2^{h4} or Kunming mice.

2. Materials and methods

2.1. Animals and specimens

NOD.H-2^{h4} mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Kunming mice were purchased from Weitonglihua Biotech in China. The mice were raised and bred in a specific pathogen-free (SPF) environment in the Experimental Animal Center at China Medical University. All experimental procedures were approved by the Experimental Animal Management Committee of China Medical University. The mean iodine concentration of the normal chow was 460 μg/kg. We randomly divided 20 male NOD.H-2^{h4} mice and 20 male Kunming mice (approximately 5 weeks of age, mean weight approximately 22 g) into normal control (CON) and high iodine (HI) groups. Mice in the HI groups were given 0.05% sodium iodide in sterile distilled water for 12 weeks, while animals in the CON groups were given sterile distilled water during the same time period. Blood was collected via the orbital vein after anesthesia was administered. Serum was separated after coagulation for 1 h and centrifugation. A median cervical incision was made, and the skin and muscle layers were removed to completely expose the trachea. The thyroid gland and adjacent trachea were excised, and the two thyroid lobes were gently dissociated on ice. After a left abdominal incision was made, the spleen was removed and washed with cold phosphate-buffered saline (PBS) to prepare a mononuclear cell suspension.

2.2. Thyroid histology staining and inflammation scoring

The thyroid tissue was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned into 4 μm slices, and stained with hematoxylin and eosin (H&E). Thyroid histological changes were investigated in the maximum cross-sectional view of the tissue at 100× magnification under a light microscope system (BX51/BX52, Olympus, Japan). Image-Pro Plus 5.1 software was used to evaluate scores of lymphocytic infiltration determined by two independent investigators, and disagreements were resolved by multiple scoring. The evaluation standards were as follows [29]: 0+, normal tissue morphology without lymphocytic infiltration; 1+, interstitial accumulation of cells between two or three follicles; 2+, 10–20 infiltrated follicles, with the lymphocytic area constituting < 10% of the total gland area; 3+, the lymphocytic area constituted > 10% but < 40% of the total gland area; 4+, the lymphocytic area constituted > 40% of the total gland area; 5+, extensive infiltration in > 80% of the total tissue.

2.3. Measurement of serum thyroglobulin antibody (TgAb) levels

Murine thyroglobulin was extracted and purified as previously described [5]. Serum TgAb levels were assayed by an indirect enzyme-linked immunosorbent assay (ELISA). In brief, a 96-well plate was coated with thyroglobulin antigen overnight at 4 °C and washed 5 times with washing buffer. Then, 0.1 ml of each serum sample at a 1:100 dilution was added to triplicate wells and incubated at 37 °C for 2 h. The samples were discarded, and the plate was washed 5 times and blocked with 5% bovine serum albumin for 20 min. Peroxidase-labeled goat anti-mouse immunoglobulin G (Sigma-Aldrich, USA) was diluted 1:1000, and 0.1 ml was added to each well and incubated at 37 °C in the dark for 30 min. Next, 0.1 ml of substrate working solution, containing tetramethylbenzidine, was added and incubated at room temperature for approximately 6 min until color change was observed. Finally, 0.1 ml of stop solution was added, and the absorbance of each well at a wavelength of 450 nm was determined using a microplate reader (Bio-Rad 680, USA).

2.4. Flow cytometry cell sorting and treatment in vitro

Spleens were gently ground on filter screens to generate a cell suspension, which was then washed 3 times with cold PBS. Mononuclear cells were separated by Ficoll-Hypaque (#DKW33-R0100, Dakewei, Beijing) density gradient centrifugation. A FITC-conjugated anti-CD3 antibody (#561827, BD Pharmingen, USA) and a PE-conjugated anti-CD19 antibody (#561736, BD Pharmingen, USA) were added and incubated at room temperature for 15 min in the dark according to the manufacturer's instructions. Cell separation was carried out on a Becton Dickinson FACS instrument (BD, USA) as soon as possible to maintain cell viability. Experienced technicians were responsible for gating. The isolated T and B subpopulations were collected and stored at − 80 °C for total DNA, RNA and protein extraction. Additionally, a small fraction of T and B lymphocytes from NOD.H-2^{h4} and Kunming mice in control groups were treated with 10 μM 5-aza-deoxycytidine (S1200, Selleckchem, Houston, TX, USA) for 72 h in vitro and served as negative controls for the detection of global DNA methylation.

2.5. DNA extraction and global DNA methylation measurement by absorptiometry

Total DNA was extracted from the separated $CD3^+$ T cells, $CD19^+$ B cells and 5-aza-deoxycytidine-treated cells with a TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China). The concentration and quality of the purified DNA dissolved in TE buffer was analyzed on a NanoDrop 2000C spectrophotometer (NanoDrop Technologies, USA), and samples with an OD260/OD280 ratio between 1.8 and 2.0 were considered acceptable and used for experiments. The global DNA methylation level was assayed by absorptiometry using a Methylamp™ Global DNA Methylation Quantification Kit (#P1014, Epigenetek Group Inc., Brooklyn, NY, USA) according to the manufacturer's instructions. In brief, 200 ng of DNA from each sample was added to a