Immunobiology 219 (2014) 602–610

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

The reduced proportion of New splenic T-cells in the zinc-deficient growing rat is not due to increased susceptibility to apoptosis

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ARTICLE INFO

Article history:
Received 1 May 2013
Received in revised form
23 September 2013
Accepted 12 March 2014
Available online 21 March 2014

Keywords:
Apoptosis
CD90
Flow cytometry
Rats
Zinc deficiency

ABSTRACT

Dietary zinc deficiency has been associated with an increased risk of infection. It has been reported that zinc-deficient rats have fewer New T-cells (TCRαβ$^+$CD90$^+$) compared to diet-restricted and control rats, which over time could adversely affect the ability of the organism to fight off infections. We hypothesized that the lower proportion of New T-cells in zinc deficiency is due to an increased susceptibility to apoptosis. Weanling, Sprague Dawley rats were assigned to one of four dietary treatment groups for 3 weeks: zinc-deficient (ZD, <1 mg zinc/kg, *ad libitum*), diet-restricted (DR, 30 mg zinc/kg, limited to the amount of feed as consumed by ZD), marginally zinc-deficient (MZD, 10 mg zinc/kg, *ad libitum*) or control (CTL, 30 mg zinc/kg, *ad libitum*). Thymocytes and splenocytes were labeled for flow cytometric determination of cell surface markers and DNA staining (for simultaneous determination of the phenotype of apoptotic cells) and assessed by Western blotting for apoptotic markers. Cells were analyzed immediately, or after incubation for 7 h with or without dexamethasone. There was no difference in the proportion of CD90$^+$ thymocytes; however ZD rats had a higher proportion of Cytotoxic (CD90$^+$4$^-$8$^+$) thymocytes compared to MZD and CTL. ZD had a lower proportion of splenic New T-cells compared to DR, MZD and CTL. There was no effect of diet on the proportion of apoptotic thymocytes or splenocytes, except ZD splenoctyes had a lower Bax/Bcl-xl ratio compared to DR and CTL. We characterized the splenic New T-cells into Helper and Cytotoxic subsets and found that ZD had a higher ratio of Helper to Cytotoxic New T-cells compared to MZD and CTL. These results do not support the hypothesis of increased apoptotic removal of New T-cells in ZD in growing rats. The regulation of CD90 expression should be explored in future studies.

Introduction

It has been estimated that zinc deficiency affects 1/3 of the world's population with children being a segment of the population at particular risk (Guilbert, 2003). Zinc deficiency has been known to impair immune function, and is the cause of approximately 10% of diarrheal disease, 16% of lower respiratory tract infections, and 18% of malaria cases (Guilbert, 2003). Studies from around the world have shown that zinc supplementation reduces the rates of diarrhea, pneumonia, malaria and mortality in children (Ninh et al., 1996; Sazawal et al., 1998; Shankar et al., 2000; Umeta et al., 2000). It has therefore been of great interest to determine why dietary zinc deficiency leaves children more susceptible to disease. Several recent reviews have addressed potential mechanisms for the immunodeficiency of zinc deficiency (Prasad, 2007; Murakami and Hirano, 2008; Haase and Rink, 2009; Blewett and Taylor, 2012; Chasapis et al., 2012; Wong and Ho, 2012).

Abbreviations: CTL, control group; CD90$^+$4$^-$8$^+$, Cytotoxic thymocytes; DAPI, 2′,6-diamidino-2-phenylindole; DEX, incubated for 7 h with dexamethasone; DR, diet-restricted group; FBS, fetal bovine serum; CD90$^+$4$^+$8$^-$, Helper thymocytes; IMM, stained immediately upon cell isolation; INC, incubated for 7 h; TCRαβ$^+$CD90$^-$, Mature T-cells; TCRαβ$^+$CD90$^-$4$^-$8$^+$, TCRαβ$^+$CD90$^-$4$^-$8$^+$; TCRαβ$^+$CD90$^-$4$^+$8$^-$, Mature Helper T-cells; MZD, marginal zinc-deficient group; TCRαβ$^+$CD90$^+$, New T-cells; TCRαβ$^+$CD90$^+$4$^-$8$^+$, New Cytotoxic T-cells; TCRαβ$^+$CD90$^+$4$^+$8$^-$, New Helper T-cells; CD90$^+$4$^+$8$^+$, Pre-T-cells; CD90$^+$4$^-$8$^-$, Pro-T-cells; TCRαβ$^+$, Total T-cells; ZD, zinc-deficient group.

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http://dx.doi.org/10.1016/j.imbio.2014.03.001
0171-2985/

Studies of zinc-deficient adult mice have shown lymphoid organ atrophy, lower T-cell numbers, impaired T-cell function and a decreased ability to fight infection compared to control mice (Shi et al., 1998). Elevated corticosterone levels have also been associated with increased apoptosis of Pre-T-cells ($CD4^+CD8^+$) and greatly reduced thymocyte numbers in zinc-deficient adult mice (King et al., 2002). King and colleagues (2002) postulate that these results explain the lymphopenia and subsequent increased vulnerability to disease in dietary zinc deficiency.

The growing rat model of dietary zinc deficiency does not show lymphopenia relative to organ weight or blood volume (Hosea et al., 2004), but they do have fewer newly released T-cells (recent thymic emigrants) compared to *ad libitum* fed controls (Hosea et al., 2003). Fewer recent thymic emigrants over time would limit the T-cell repertoire, which is another possible explanation for the increased susceptibility to infection associated with dietary zinc deficiency. CD90 first appears on cortical thymocytes and is found on recent thymic emigrants for approximately 3 days in rats (Hosseinzadeh and Goldschneider, 1993). A greater susceptibility of recent thymic emigrants to apoptosis has been reported in the diabetes-prone BB rat (Iwakoshi et al., 1998), which are similar to zinc deficient rats in that they have reduced lymphocyte numbers compared to controls, and the proportion of T-cell subsets using CD4 and CD8 labeling remains unchanged (Jung et al., 1999).

We hypothesized that the reduced proportion of recent thymic emigrant T-cells in zinc-deficient rats is due to increased susceptibility of $CD90^+$ T-cells to apoptosis upon exiting the thymus. Flow cytometry can be used to quantify apoptosis in specific cell populations by simultaneously staining cell surface markers and DNA to generate cell cycle profiles (Fraker et al., 1995). The cells that appear in the sub-G1 area of the cell cycle are apoptotic. In the present experiment, we used flow cytometry and four or five-color labeling of thymic and splenic cells for characterization of T-cell phenotypes and to determine proportion of apoptosis in T-cell sub-populations among zinc-deficient, diet-restricted, marginally zinc-deficient and control growing rats. We also investigated apoptotic proteins (Bcl-xL (pro-survival), Bcl-2 (pro-survival), Bax (pro-apoptotic), caspase 3 (cleaved caspase 3 for activation of caspase 3 (Xiao et al., 2010)) by Western blotting in thymoctyes and splenocytes.

Materials and methods

Animals and diets

Weanling Sprague Dawley rats (Charles River Laboratories, St. Constant, PQ) were acclimatized for 5 days and randomly assigned to one of four dietary treatment groups for three weeks: zinc-deficient (ZD, <1 mg zinc/kg diet), marginally zinc-deficient (MZD, 10 mg zinc/kg diet), nutritionally complete control diet (CTL, 30 mg zinc/kg diet), and diet-restricted (fed the control diet, but only the amount of feed as consumed on the previous day by the individual zinc deficient rat paired to the DR rat). The diet-restricted group controls for the effects of undernutrition commonly seen in zinc deficiency. Body weight was measured weekly and feed intake was measured daily.

The experimental diets (based on the American Institute of Nutrition-93G formulation and previously described by Lepage et al. (1999) containing egg white, additional biotin (2 mg/kg diet) and potassium phosphate were fed *ad libitum*, except for the DR group, and distilled water was provided. Zinc content of the diets was verified by atomic absorption analysis. To avoid zinc recycling and contamination, the rats were housed individually in stainless steel hanging cages with mesh bottoms so urine and feces could drop to the shavings below. In addition, the ZD group was placed on the upper rows of the cage rack. The rats were maintained in an environment of controlled temperature (21–23 °C), humidity (55%) and light cycle (14 h light/10 h dark). Animal care was provided in accordance with a protocol approved by the University of Manitoba Protocol Management and Review Committee.

At the end of the feeding trial, the rats were euthanized by CO_2 asphyxiation and decapitation. Trunk blood was collected and centrifuged to obtain serum and stored at −80 °C until analysis. The spleen and thymus were removed aseptically, weighed and processed immediately. Femurs were removed, cleaned of soft tissue and stored at −20 °C until analysis.

Zinc analysis

After obtaining wet and dry weights, femurs and diet samples were wet-ashed using trace element grade nitric acid. After appropriate dilution of digests or serum, zinc concentration was determined by atomic absorption spectrometry (Varian Spectra AA, Varian Australia, Mulgrave, VI). Bovine liver standard reference material 1577b (US Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD) was used as quality control.

Cell preparation

Single cell suspensions of spleen and thymus were prepared by pressing tissues through nylon screens into Hank's buffered saline supplemented with 10 mM HEPES, 4% dextran charcoal absorbed fetal bovine serum (FBS; Hyclone, Logan, UT), and 1% antibiotic/antimycotic at pH 7.4. Red blood cells from the spleen cell suspensions were lyzed using ammonium chloride lysis buffer (155 mM NH_4Cl, 0.1 mM EDTA, 10 mM $KHCO_3$, pH 7.2). Cell concentration and viability were determined using trypan blue dye exclusion on an AO Bright-Line Hemacytometer (American Optical Corporation, Buffalo, NY).

Determination of T-lymphocyte subpopulations

Antibodies

Monoclonal antibodies for TCRαβ (PE label, R73 clone, isotype mouse $IgG_{1,k}$), CD90 (FITC label, Thy1.1, clone OX-7 isotype, mouse $IgG_{1,k}$), CD4 (PE-Cy5 label, OX-35 clone, isotype mouse $IgG_{2a,k}$), and CD8 (biotin, OX-8 clone, isotype mouse $IgG_{1,k}$) were obtained from BD pharmingen (Mississauga, ON). Streptavidin-PE-Cy7 conjugate was used to label the biotinylated antibodies. 2′,6-Diamidino-2-phenylindole (DAPI, 0.2 μmol/L) was used to stain DNA to generate cell cycle data.

Cell labeling and flow cytometry

Thymocytes and splenocytes were subjected to three *in vitro* conditions. Because apoptotic cells are removed by phagocytes, it can be difficult to study apoptotic cells *in vivo*. In the present study we analyzed cells immediately (IMM) to determine the phenotype immediately upon cell isolation, or the cells were cultured for 7 h (INC) to allow the cells that were stimulated *in vivo* to undergo apoptosis to appear in the relative absence of phagocytes, or the cells were incubated for 7 h with dexamethasone (DEX) to initiate apoptosis and determine whether dietary treatment influenced the susceptibility of thymocytes and splenocytes to apoptosis *in vitro*. Cells (1×10^6) from each dietary treatment group and tissue were incubated with fluorescent antibodies immediately, while the remaining cells were resuspended in culture medium (RPMI-1640 containing 10 mM HEPES, 10 mM sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 μM 2-mercaptoethanol, and 5% FBS (Sigma, St. Louis, MO)) and cultured for 7 h at 37 °C with 5% CO_2 in the absence or presence of 1 μmol/L DEX. The DEX-treated cells were a positive control for