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Effects of hexavalent chromium on mouse splenic T lymphocytes

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ABSTRACT

Hexavalent chromium [Cr(VI)] is widely used in various industrial processes and has been recognized as a carcinogen. As the first line of host defense system, the immune system can be a primary target of Cr(VI). T cell population represents a major arm of the immune system that plays a critical role in host anti-tumor immunity. Dysfunction of T cells, such as exhaustion under the persistent presence of tumor antigen, compromise host anti-tumor immunity resulting in oncogenesis. Previous studies have shown Cr(VI) exposure alters the phenotype of human peripheral blood lymphocytes. However, the mechanism of the alteration and whether such an alteration in immunity affects immunosurveillance and promotes carcinogenicity are not clear. Using a culture of mouse splenic T cells as an in vitro model system, the present study assessed the effects of Cr(VI) on T cells, as the first step in our investigation of the mechanism underlying Cr(VI)-inhibited immunosurveillance and carcinogenesis. Our results showed that Cr(VI) decreased the viability of $CD4^+$ and $CD8^+$ T cells and inhibited their activation, proliferation, cytokine release and cytolytic function.

1. Introduction

Chromium(Cr) exists widely in nature and is used in various industrial processes (Barceloux, 1999). Humans can be exposed to chromium through inhalation, ingestion, or dermal exposure (Shrivastava et al., 2002). In spite of 6 valence states of chromium, only Cr(III) and Cr(VI) are of biological significance with respect to their toxicity and carcinogenicity (Steinhagen et al., 2004). Cr(VI) is considered a human carcinogen and chronic exposure to Cr(VI) is associated with increased incidence of various cancers (Costa, 1997; Salnikow and Zhitkovich, 2008).

The immune system is an interactive network of lymphoid organs, cells, humoral factors and cytokines, which together act as the first line of host defense against infection or cancer (Candeias and Gaipl, 2016; Parkin and Cohen, 2001; Subramanian et al., 2015). The immune system monitors the host body, recognizing and eliminating newly arising cancer cells to stop/control tumor formation in a process termed 'immunosurveillance'. Cancer cells possess tumor antigens. The encounter between cancer cells and immune system initiates a process known as 'immunoediting' that can bring about three outcomes: elimination, equilibrium or escape of cancer cells from immunosurveillance. Hence, the host immunity suppresses tumor development, whereas tumor formation implicates a successful escape of tumor cells from host immunosurveillance. Previous studies have shown that Cr(VI) may impair the immune system (Akbar et al., 2011; Dangleben et al., 2013; Salsano et al., 2004) and occupational exposure to Cr(VI) decreased the number of lymphocytes in peripheral blood of chromate workers (Qian et al., 2013; Tanigawa et al., 1995). However, the effects of Cr(VI) on host anti-tumor immunity is not clear.

The host anti-tumor immunity can be carried out in a form of cell-mediated immunity. As an important component of cell-mediated immunity, T cells play a critical role in immunosurveillance (Williams and Bevan, 2007). $CD4^+$ and $CD8^+$ are two major T cell subpopulations. Quiescent naïve T cells mainly reside in secondary lymphoid organs such as the spleen (Mebius and Kraal, 2005). Upon being attacked by either exogenous or endogenous insult, T cells are activated and undergo rapid clonal expansion (proliferation). Activated $CD4^+$ T cells perform their effector functions through secreting cytokines, whereas activated $CD8^+$ T cells mediate lysis of target cells via releasing perforin and granules (Broere et al., 2011). Due to a high content of T cells, the culture of mouse splenocytes has been widely used as an in vitro model system to test immunotoxicity to T cells by a variety of insults (Pestka et al., 1994; Song et al., 2014).

Our long-term goal is to understand the effects of Cr(VI) on host

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anti-tumor immunity and its contribution to Cr(VI) carcinogenicity. As the first step, in the current study we aimed to determine whether Cr (VI) at environmental relevant concentrations affects the viability, activation, proliferation and effector function of T cells. Using the culture of mouse splenocytes as an in vitro model, our results showed that Cr (VI) decreased the viability of $CD4^+$ and $CD8^+$ T cells, inhibited the activation, proliferation, cytokine secretion and cytolytic function of the T cells. These results set up a foundation for our further investigation of the mechanism underlying Cr(VI)-induced immunosurveillance inhibition and carcinogenesis.

2. Materials and methods

2.1. Animals

Male and female C57BL/6 mice (8-week-old) were purchased from Harlan Laboratory (Indianapolis, IN) and maintained under specific pathogen-free conditions at the Division of Laboratory Animal Resources, University of Kentucky Medical Center (Lexington, KY). All protocols were in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

2.2. Chemicals

Potassium dichromate ($K_2Cr_2O_7$) was obtained from Sigma-Aldrich (St. Louis, MO) and used for Cr(VI) treatment. RPMI-1640 medium and fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY). Anti-CD3/CD28, APC-conjugated anti-CD4, PE-conjugated anti-CD4, PE-Cy5.5-conjugated anti-CD4, APC-conjugated anti-CD8, FITC-conjugated anti-CD8, PE-conjugated anti-69, FITC-conjugated anti-CD25, FTIC-conjugated CD107a were from BioLegend (San Diego, CA). Mouse IL-2, IL-4, IL-10 ELISA MAX™ Deluxe Set and carboxyfluorescein diacetate succinimidyl ester (CFSE) cell division tracker kit were obtained from BioLegend (San Diego, CA).

2.3. Splenocyte cultures

C57BL/6 mice were sacrificed and their spleens were aseptically removed. A suspension of splenocytes was obtained by meshing the spleen through a cell strainer (Corning Incorporated, Durham, NC). Erythrocytes were lysed in RBC lysis buffer (10 ×) (BioLegend, San Diego, CA), and the cell pellets were washed with RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml of penicillin and streptomycin, and 0.1% β-mercaptoethanol. The cell number was assessed by exclusion of trypan blue (Sigma-Aldrich, St. Louis, MO). Cells were plated at a density of 6×10^6 cells/ml and maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

2.4. Cell viability assay

T cell viability was determined via propidium iodide (PI) staining assay. PI is impermeable to intact plasma membranes of living cells, allowing the accurate determination of cell viability in a variety of studies (Bank, 1987; Yeh et al., 1981). In this experiment, the isolated splenocytes were plated on 24-well plates (Costar, Washington) containing 1 ml of RPMI-1640 complete medium. Then cells were treated with 0, 2 or 5 μM Cr(VI). After 24 h, cells were washed and re-suspended in 0.5 ml FACS buffer (PBS, 2% FBS and 0.1% sodium azide). Single cell suspensions were labeled with APC-conjugated anti-CD4 and FITC-conjugated anti-CD8 antibodies on ice for 30 min in the dark. Shortly before flow cytometric analysis, 20 μl of the PI solution was added to 0.5 ml of cell suspension. Then, cells were analyzed with BD FACSCalibur flow cytometry (San Jose, California). Three independent experiments were conducted and each treatment contained triplicates.

2.5. T cell activation assay

Anti-CD3 and anti-CD28 antibodies were used to induce T cell activation (Frauwirth and Thompson, 2002) which can be detected by the increase of CD69 and CD25 levels on T cell surface (Morzadec et al., 2012). Briefly, splenocytes were treated with anti-CD3 and CD28 antibodies (1 μg/ml) for 24 h. Cells were stained with APC-conjugated anti-CD4, anti-CD8, PE-conjugated anti-CD69 and FITC-conjugated anti-CD25 antibodies. After 30 min of staining, cells were analyzed using BD FACSCalibur flow cytometry. PI staining was used to exclude dead cells.

2.6. T cell proliferation assay

The proliferation of splenic T cells was measured as previously described (Quah and Parish, 2010). Briefly, the intracellular fluorescent dye, CFSE, covalently labels intracellular molecules. With each cell division, these fluorescent molecules are equally distributed in daughter cells, shown as the half-reduced CFSE intensity in daughter cells, which allows T cells proliferation to be examined by flow cytometry (Quah and Parish, 2010). Therefore, the splenocytes were labeled with CFSE and cultured in a 24-well plate in medium with or without anti-CD3/CD28 antibodies (1 μg/ml). Cells were then treated with 0, 2 or 5 μM Cr(VI) for 96 h and stained with PE-Cy5.5 conjugated anti-CD4 and APC-conjugated anti-CD8 antibodies, and cell proliferation was analyzed by flow cytometry. PI staining was used to exclude dead cells.

2.7. Determination of cytokine levels

The cytokine levels were determined using ELISA kits as described previously (Katial et al., 1998). Splenocytes were cultured in 24-well plates and treated with either anti-CD3/CD28 antibodies alone or anti-CD3/CD28 antibodies plus Cr(VI) (2 or 5 μM) for 24 h. Cell-free supernatants were collected for measurement of cytokine levels by ELISA.

2.8. Detection of the cytolytic function of $CD8^+$ T cells

As previously described, $CD8^+$ T cell mediated cytotoxicity was evaluated via measuring the levels of CD107a which is expressed on T cell surface following activation-induced degranulation (Betts et al., 2003). Briefly, 1×10^6 cells were cultured in complete RPMI and treated with 2 or 5 μM Cr(VI). After 18 h, anti-CD3/CD28, FTIC conjugated CD-107a or hamster IgG isotype control antibodies were added. One hour later, 1 μl monensin solution (1000 ×) was added to each well. Cells were then cultured in a humidified atmosphere of 5% CO^2 at 37 °C for an additional 5 h and then were stained with APC-conjugated CD8 antibodies. The expression of CD107a on T cell surface was detected by flow cytometry. Three independent experiments were conducted with triplicates for each treatment group.

2.9. Statistical analysis

All experiments were repeated at least three times with triplicates. Data were expressed as mean ± standard deviation. GraphPad Prism was used to analyze all data. Comparison of more than two groups was made with a one-way analysis of variance (ANOVA) followed by Tukey's test. A P value < 0.05 was considered significant.

3. Results

3.1. Cr(VI) decreases T cell viability

We first determined the effects of Cr(VI) on the viability of $CD4^+$ and $CD8^+$ T cells. The splenocytes were treated with 2 or 5 μM Cr(VI) in vitro and the viability of $CD4^+$ and $CD8^+$ T cells in the culture was determined by PI staining followed by flow cytometry. Lymphocytes