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Arsenic trioxide is an immune adjuvant in liver cancer treatment

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ABSTRACT

Tumor cells are inhibited effectively by As_2O_3 *in vitro* and *in vivo*, although the underlying immune regulatory mechanisms remain unknown. Regulatory T cells play a key role in tumor immune escape. In the present study, we aimed to assess the *in vivo* effects of As_2O_3 on the immune status in hepatic cancer and its *in vitro* regulatory role in cytokine-induced killers(CIKs)cytotoxicity. In a tumor H22 xenograft model of hepatic cancer, we demonstrated that As_2O_3 treatment decreased tumor volumes and weights, and improved survival by reducing Tregs infiltration into the tumor. Moreover, our data indicated that the exact immune regulatory mechanism of As_2O_3 might involve elevated CD3 + T lymphocyte amounts more than reduced Tregs levels. Furthermore, As_2O_3 significantly improved CIKs cytotoxicity *in vitro* by decreasing CD4 + T lymphocytes and Tregs, and increasing CD8 + T lymphocytes. Our results suggested that As_2O_3 might act as an immune adjuvant in liver carcinoma treatment by increasing T lymphocytes and decreasing Treg infiltrated into the tumor.

1. Introduction

Studies assessing As_2O_3 are widely carried out in solid tumors such as liver (Alarifi et al., 2013; Zhang et al., 2012) and gastrointestinal (Lee et al., 2013) carcinomas, while preclinical studies found it effective in leukemia as well. As a powerful inducer of oxidative stress in tumor cells, As_2O_3 applied solely in practice is not therapeutic (Chen et al., 2009; Lin et al., 2007). However, the antitumor effects of certain treatment modalities, including Chinese medicine preparations (Li et al., 2014b; Zhao et al., 2014), chemotherapeutic drugs (Rangwala et al., 2012; Zhai et al., 2015) and local regional therapy (Wang et al., 2015) are enhanced when combined with As_2O_3, indicating that As_2O_3 might contribute in treating tumors with yet unknown mechanisms.

Recently, the immunomodulatory properties of As_2O_3 were described. Indeed, preclinical studies showed that As_2O_3 affects peripheral blood leukocyte and platelet counts (Jiang et al., 2010; Xu et al., 2004), which may ultimately alter the immune system. In addition, As_2O_3 does not only increase the susceptibility of breast cancer cells to LAKs (lymphokine-activated killers), but also enhances the lytic efficiency of these cells(Baj et al., 2002). Furthermore, As_2O_3 enhances antitumor immunity in myeloma models (Ge et al., 2009) and breast cancer cells (Baj et al.,, 2002), although the exact mechanisms remain unclear. Subsequently, studies demonstrated that As_2O_3 induces apoptosis of CD4 + T cells by upregulating C/EBP homologous protein and glucose regulated protein78 (Li et al., 2013) or activating the Fas-FasL pathway(Liao et al., 2009), with no obvious effects on the CD8 + T cell subpopulation (Soto-Pena et al., 2006; Thomas-Schoemann et al., 2012). Moreover, As_2O_3 selectively regulates the proliferation of Treg rather than non-Treg CD4 T cells *in vitro*, in a dose dependent manner; however, disparate results were obtained between humans and mice (Hernandez-Castro et al., 2009; Thomas-Schoemann et al., 2012).

Regulatory T cells (Tregs), as a group of suppressive T cells, play a key role in tumor immune escape (Huang et al., 2014; Lavotshkin et al., 2015; Li et al., 2014a). However, mounting evidence from clinical studies demonstrates that local Tregs infiltration could not be considered an independent factor affecting cancer prognosis (Kim et al., 2015; Nakagawa et al., 2015). Correspondingly, the ratio of CD3 + T to Tregs substituting for Tregs alone is a widely accepted

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independent factor of tumor prognosis (Lavotshkin et al., 2015; Mathai et al., 2012).

This study aimed to assess whether As_2O_3 could reduce the proportion of Tregs in the pool of CIKs, while enhancing their lytic activity *in vitro*. In addition, the exact *in vivo* antitumor immune mechanism of As_2O_3 was explored.

2. Materials and methods

2.1. Ethics statement

Written informed consent was obtained from the patients and healthy donors. This study was approved by the Ethics Committee of The Zhongnan Hospital, Wuhan University. The experimental mice used in this study were obtained from the Laboratory Animal Center of the Academy of Military Medical Science. All procedures and postoperative animal care were conducted according to protocols approved by the Animal Care and Use Committee of Chinese PLA General Hospital and the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals.

2.2. Reagents and cell lines

As_2O_3 was purchased from Sigma (St. Louis, MO, USA) and stored at 4 °C. The mouse hepatic cancer cell line H22 and human hepatic carcinoma HepG2 cells were obtained from the Beijing Institute of General Surgery, Chinese PLA General Hospital (Beijing, China). H22 cells were proliferated in the abdominal cavity of KM mouse, and collected in the logarithmic phase.

2.3. Animal models and in vivo experiments

Male KM mice, 6–8 weeks old, were purchased from the Beijing Experimental Animal Center of the Academy of Military Medical Sciences (Beijing, China). 5×10^6 H22 cells were implanted subcutaneously in left lower limbs. Tumor tissues were dissected one week later, and cut into pieces of about 0.2 mm^3 each. Mouse were anesthetized by intraperitoneal injection of pentobarbital sodium; then, a midline incision was made in the upper abdomen beneath the xiphoid process, with bilateral costal arches pressed gently, and the hepatic lobes exposed. Tumor pieces were implanted into the livers along the macroaxis (Chen et al., 2016). Tumor-bearing mice were treated with saline or As_2O_3 at 2 mg/kg daily, As_2O_3 at 4 mg/kg daily or As_2O_3 at 6 mg/kg every other day, for 2 weeks. Each group contained six mice, whose weights were recorded daily. Afterwards, mononuclear cell suspensions were prepared from blood samples collected by retro-orbital bleeding. Then, the livers were removed in sterile conditions to calculate liver to body weight ratios; the tumors extracted from the livers were evaluated for volume and weight, respectively; the spleens were removed to calculate the spleen index(SI), defined as the ratio of spleen weight(mg) to body weight(g) which was often used to reflected the immune function partially. Mononuclear cell suspensions were prepared from spleens. Peripheral blood and spleen samples were analyzed by FCM to determine the proportions of CD3 + T cells and Treg. A portion of tumor specimens was processed for H&E staining and immunofluorescence staining for CD3 and Foxp3. Another batch of mice was used to evaluate the effects of As_2O_3 on KM mouse survival.

2.4. H&E staining and immunofluorescence

Heart, liver, kidney, tail and liver tumors were obtained from mice after treatment, and fixed with formaldehyde (37%), paraffin embedded, and sliced. The degrees of tumor necrosis and local inflammatory cell infiltration were analyzed after H&E staining. CD3+ T cells and Foxp3+ Treg infiltrated in liver tumors were assessed by immunofluorescence using anti-CD3 (Abcam – ab54501, England, UK) and anti-Foxp3 (Abcam – ab36607, England, UK) antibodies, which were conjugated to green fluorescence protein (GFP). The Image-Pro Plus 6.0 software was used to convert fluorescent images to black and white pictures, used for integrated optical density (IOD) assessment, which indicated the immunofluorescence intensity of the tissue.

2.5. CIKs preparation and in vitro assays

Mononuclear cell suspensions from 20 mL of healthy human peripheral blood were prepared by density gradient centrifugation using the human lymphocyte separation medium (Chinese Academy of Medical Sciences, Beijing, China). After incubation for 3 h, non-adherent floating cells were collected and resuspended to 2.0×10^6/mL. PBMCs were seeded into 24-well plates with 1 mL of Cellix 601 serum-free medium (Beijing Xin Ming Thai Biotechnology, Beijing, China) supplemented with 10 ng/mL PHA (RD-CAA3169, MN, USA) and 500 IU/mL rhIL-2 (RD-P60568, MN, USA)(Yan et al., 2014), and treated with PBS, and 0.1 μM, 1 μM and 5 μM As_2O_3, respectively, at 37 °C in a humidified incubator containing 5% CO_2 for 48 h. Viable cells were assessed by trypan blue staining, and the proportions of CD3 + T cells, CD3 + CD4 + T cells, CD3 + CD8 + T cells, CD3-CD56 + NK cells, CD3 + CD56 + NK cells and Treg in PBMCs were determined by flow cytometry. Foxp3 gene expression levels in CIKs were detected by RT-PCR. Enzyme-linked immunoassay (ELISA) was used to assess IFN-γ amounts in cell culture supernatants. L*actate dehydrogenase* (LDH) assay was used to assess the cytotoxic activity of CIKs.

2.6. Flow cytometry (FCM)

The phenotypes of As_2O_3-treated human CIKs were characterized by FCM using antibodies against CD3, CD4, CD8, CD56, CD25, and Foxp3 (Becton Dickinson, San Diego, USA). The ratios of CD3+ (T cells) and CD4 + CD25 + Foxp3+ (Treg) were detected using antibodies raised against CD3, CD4, CD25, and Foxp3(Miltenyi Biotec, Cologne, German) (Yan et al., 2014).

2.7. Enzyme-linked immunosorbent assay (ELISA)

IFN-γ, TGF-β and IL-10 levels in serum samples were determined with ELISA kits specific to IFN-γ (Alpco-61-IFGMS-E01, NH, USA), TGF-β(Alpco-61-TB1MS-E01, NH, USA)and IL-10 (eBioscience −E17771-104, CA, USA), according to the manufacturers' protocols. Absorbance was read on a microplate reader at 492 nm (Zhou et al., 2014).

2.8. Cytotoxicity assay

In vitro cytotoxicity of CIKs towards HepG2 cells was assessed using an LDH assay kit (Sigma-Aldrich, St. Louis, MO, USA), as described previously. The target Hepg2 cells were mixed with CIKs treated with different concentrations of As_2O_3 at a ratio of 5:1 or 10:1, and cultured for 24 h. Optical density (OD) values were obtained on a microplate reader at 492 nm. Cytotoxicity was derived according to the following formula: Cytotoxicity (%) = [OD(Experimental) − OD(Effector spontaneous) − OD(Target spontaneous)] × 100/[OD(Target maximum) − OD(Target spontaneous)](Yan et al., 2014).