Downregulation of B7-H4 in the MHCC97-H hepatocellular carcinoma cell line by arsenic trioxide

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Abstract. Arsenic trioxide (As2O3; ATO), a compound which is characterized by its ability to function as a potent anticancer agent, has been investigated in a variety of carcinomas. B7-H4, a transmembrane protein, may inhibit the function of the T cell effector, and therefore, may be useful in investigating different types of tumor therapies. However, few studies have been published previously associated with the roles of ATO and B7-H4 in human hepatocellular carcinoma (HCC). The aim of the present study was to investigate the anti-invasive role of ATO in HCC, to determine the effect of ATO treatment on the expression of B7-H4 and to further assess the possible underlying mechanisms. Following treatment of the cells with 2, 4 and 8 μM ATO for 48 h, cell counting kit-8 (CCK-8), Transwell and western blot assays were used to determine the extent of human MHCC97-H HCC cell proliferation, apoptosis, invasion and B7-H4 expression, respectively. The results revealed that 1 μM ATO markedly decreased cellular proliferation, and ATO administered at concentrations of 0.1, 0.2 and 0.5 μM markedly inhibited the migration and invasion of the human MHCC97-H HCC cell line. The expression of B7-H4 in the treatment groups was markedly reduced. Signal transduction mediated via the Janus kinase 2/signal transducers and activators of transcription 3 pathway was inhibited upon treatment with 0.1, 0.2 and 0.5 μM ATO. Additionally, the protein expression levels of matrix metalloproteinase 2 and vascular endothelial growth factor were markedly reduced in HCC cells upon treatment with ATO. In conclusion, ATO may reduce the protein expression levels of B7-H4 in MHCC97-H HCC cells, and further affected HCC tumorigenesis and progression. ATO may be a putative agent for the development of therapeutic strategies against human liver cancer.

Introduction

Hepatocellular carcinoma (HCC), which is the carcinoma associated with the third highest level of mortality following gastric and esophageal cancer, is the focus of numerous previous studies aimed at investigating its etiopathogenesis and molecular therapy (1-3). HCC routinely spreads to the lymph nodes, lungs, adrenal glands and bone. A total of at least 560,000 individuals develop liver cancer annually, of which ~550,000 people consequently succumb to the disease (4,5). The majority of cases of HCC occur in Africa or in Eastern Asia. In China, the incidence of HCC accounts for >50% of the cases reported worldwide. Surgical resection and chemoradiotherapy are common treatments for HCC; however, the surgical resection procedure is limited by the number of resectable patients available, since only ~15% of patients exhibit resectable disease. Chemoradiotherapy, due to its high toxicity, causes a series of side effects, including nausea, vomiting and diarrhea, which severely damage liver function. Consequenly, there is a pressing requirement to understand the mechanism and molecular therapy of HCC.

From the 1700s through to the early 1900s, arsenic trioxide (As2O3; ATO) was known as a natural compound, which has been widely used in the treatment of leukemia. ATO regulates several cellular functions, including cell proliferation, differentiation, angiogenesis and apoptosis in diverse cell lines (6). Previous studies revealed that ATO induced apoptosis and suppressed tumor progression in several tumor cell lines, including acute myeloid leukemia, multiple myeloma, colon, liver, kidney, bladder and gastric carcinoma (7-10). The mechanism of the anticancer effects exerted by ATO remain to be fully elucidated. There is mounting evidence to suggest that the efficacy of ATO is dependent on numerous mechanisms, which are predominantly associated with apoptosis and differentiation. However, the detailed effects of ATO on HCC cell invasion remain to be elucidated.
B7-H4, a transmembrane protein, binds to an unknown receptor on activated T cells, resulting in the inhibition of T cell effector function via cell cycle arrest, decreased proliferation and reduced interleukin 2 production (11). B7-H4 is upregulated on the surface of cancer cells and immunosuppressive tumor-associated macrophages in a variety of human cancer types (12-14). B7-H4 is a promising candidate for therapeutic intervention strategies, since its expression levels are inversely correlated with patient survival rate in various types of cancer. Previous studies demonstrated that the expression of B7-H4 promoted tumorigenesis in ovarian cancer (15), non-small cell lung cancer (16), gastric and cervical cancer (11), and other types of tumor (13,17).

In the present study, the expression levels of B7-H4 in ATO-treated human MHCC97-H HCC cells, and the anti-invasion and anti-invasion effects of ATO, were investigated. In addition, the possible mechanism(s) involved was also investigated.

Materials and methods

Patients and tissue samples. A total of 30 patients with liver cancer at stage II-III, who had been admitted to The Second Affiliated Hospital of Harbin Medical University (Harbin, China) between January 2009 and December 2009, were enrolled in the present study. All patients had complete clinical and pathological follow-up data, and the adjacent normal liver tissues were also collected as negative controls. Liver cancer tissues and normal liver tissue resected within at least 5 cm of the tumor margin, were collected when the patients underwent definitive surgery. Ethical approval for the present study was provided by the independent ethics committee of the Second Affiliated Hospital of Harbin Medical University. Informed and written consent was obtained from all patients or their advisers, according to the ethics committee guidelines.

Cell culture and treatment. Human MHCC97-H liver carcinoma cells were obtained from American Type Culture Collection (Manassas, VA, USA), and the cells were subsequently cultured in RPMI-1640 medium (HyClone Laboratories, Logan, UT, USA), supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 50 U/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc.) and 50 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), and were maintained at 37˚C with 5% CO₂ in a sterile incubator.

Cell counting kit-8 (CCK-8) assay. The proliferation of the MHCC97-H cells was assessed using a CCK-8 kit, purchased from JRDUN Biotechnology (Shanghai, China). Briefly, cells were suspended in 100 ml RPMI-1640 medium from either control untreated cells or from cells which were treated with different doses (0.1, 0.2 or 0.5 μM) of ATO (Harbin Pharmaceutical Group Medicine Co., Ltd., Harbin, China). The MHCC97-H cells were seeded at a density of 2x10⁴/well into a 96-well plate and were subsequently incubated at 37˚C in a humidified atmosphere containing 5% CO₂ for 48 h. The optical density values were measured using a SpectraMAX microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 450 nm. The experiment was repeated three times, and each experiment was performed with six replicates. The extent of cellular proliferation was determined, according to the manufacturer's instructions.

Cell cycle distribution. Following treatment with ATO (0.1, 0.2 or 0.5 μM) for 24 h, the cells were treated with trypsin (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China), centrifuged at 1,000 x g for 5 min, washed with ice-cold phosphate-buffered saline (PBS), fixed in 70% ethanol and stored at 4˚C. The pellets were subsequently collected, washed twice with PBS and stained with propidium iodide (Sigma-Aldrich, Munich, Germany) in the presence of RNaseA (Sigma-Aldrich) for 45 min in the dark. The cell cycle distribution was analyzed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) with BD CellQuest™ software 3.0 (BD Biosciences).

Cell migration assay. Human MHCC97-H cells were seeded into a six-well plate (1x10⁴ cells/well). At 24 h following treatment with ATO (0.1, 0.2 or 0.5 μM), the medium was changed to serum-free RPMI-1640, and the cells were incubated for 12 h. Following the subsequent removal of the media from the wells, a straight transverse line through the adherent cells was drawn using a ruler and a 1,000 μl tip, resulting in a uniform gap. The medium was changed to RPMI-1640, supplemented with 2% fetal bovine serum. Following a further 24 h incubation, the distances between the gaps were measured in centimeters following capture of six random sites in the microscope fields (ECLIPSE Ti-S, Nikon Corporation, Tokyo, Japan).

Figure 1. Expression levels of B7-H7 in cancer and normal tissues. The expression level of B7-H4 was detected by reverse transcription-quantitative polymerase chain reaction in 75 lung cancer tissues and their adjacent normal tissues. P<0.01, tumor, vs. normal tissue.

Matrigel invasion assay. To assess liver carcinoma cell invasion, the cells were assayed using a 24-well Transwell chamber with a pore size of 8 μm (Corning Incorporated, Corning, NY, USA). The cells were treated with trypsin following treatment with control or ATO for 48 h, and transferred to the upper Matrigel chamber in 100 μl serum-free medium, containing 1x10⁵ cells prior to a further 24 h incubation. The lower chamber was filled with medium, containing 10% fetal bovine serum as a chemoattractant. Following incubation, the non-invaded cells on the upper membrane surface were removed with a cotton tip, and the cells, which passed through the filter were fixed and stained with 0.1% crystal violet (Beijing Solarbio...
Science and Technology Co., Ltd.). The number of invaded cells was counted in five randomly selected high-power fields under a microscope (Nikon). The experiment was replicated three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Human MHCC97-H liver carcinoma cells were seeded at a density of 5x10^5 cells/well in six-well plates, cultured overnight and were subsequently treated with ATO (0, 0.1, 0.2 and 0.5 µM) for 12 h. The total RNA from ATO-treated cells was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The RT-qPCR reactions were performed using 2 µg total RNA with a first-strand cDNA kit (Sigma-Aldrich), according to the manufacturer's instructions. PCR amplification was performed for 10 min at 95˚C, followed by 40 cycles at 95˚C for 15 sec and annealing/extension at 60˚C for 45 sec in an ABI 7300 Thermocycler (Applied Biosystems, Foster City, CA, USA), using the SYBR Premix ExTaq kit (Takara Bio., Inc., Dalian, China). The specific primer sequences for each gene (Generay Biotech Co., Ltd., Shanghai, China) were as follows: B7-H4, forward: 5’-AGGGAGTGGAGGGAGATACAG-3’ and reverse: 5’-GCAGCACCCAAAGAGACAG-3’ (product, 245 bp); vascular endothelial growth factor (VEGF), forward: 5’-TCGAGACCTTGTTGACATC-3’ and reverse: 5’-CACACGGACGGCTTGAAGA-3’ (product, 71 bp); matrix metalloproteinase 2 (MMP2), forward: 5’-TTGACGGTGAAAGACGACTC-3’ and reverse: 5’-GGCGTTCACATTTCCACAC-3’ (product, 134 bp); GAPDH, forward: 5’-CACCCACTCTCCACCTTTG-3’ and reverse: 5’-CCACCACCTGTTGC TGTAG-3’ (product, 110 bp). Data analysis was performed using the 2^ΔΔCt method for quantification (18), and all samples were normalized against GAPDH, which was used as an endogenous control.

Western blot analysis. Equal quantities (30-40 µg) of protein lysates were separated by 10% SDS-PAGE and were transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% milk and probed with the following specific primary antibodies: Rabbit polyclonal B7-H4 polyclonal (1:500; Abcam, Cambridge, MA, USA; ab176146), rabbit anti-human polyclonal phosphorylated (p)-signal transducers and activators of transcription 3 (p-STAT3; 1:500; Abcam; cat. no. ab30647), mouse anti-human monoclonal STAT-3 (1:100; Abcam; cat. no. ab50761), mouse anti-human monoclonal MMP2 (1:2,000; Abcam; cat. no. ab2462), rabbit anti-human polyclonal VEGF (1:1,000; Abcam; cat. no. ab46154), rabbit anti-human monoclonal p-Janus kinase 2 (p-JAK2; 1:1,000; Cell Signaling Technology, Danvers, MA, USA; cat. no. 8082), rabbit anti-human monoclonal JAK2 (1:1,000; Cell Signaling Technology; cat. no. 3230) and rabbit monoclonal GAPDH (1:1,500; Cell Signaling Technology; cat. no. 5174). The blots were subsequently washed with Tris-buffered saline with 0.05% Tween-20 and then incubated with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA) and visualized by enhanced chemiluminescence (EMD Millipore).
Statistical analysis. The SPSS 16.0 software system (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The data are expressed as the mean ± standard error of the mean. The differences between the groups were analyzed using Student’s t-test when comparisons were made between only two groups and one-way analysis of variance was used when >2 groups were compared. All tests performed were two-sided. P<0.05 was considered to indicate a statistically significant difference.

Results

High protein expression levels of B7-H4 are associated with human liver carcinoma. To determine the biological role of B7-H4 in human liver cancer, RT-qPCR was used to detect the expression levels of B7-H4 in the tissues of patients with liver cancer. A total of 30 liver cancer tissues and their adjacent normal tissues were collected. As shown in Fig. 1, the expression levels of B7-H4 were higher in liver tumor tissues compared with the adjacent normal tissue control (P<0.001).

ATO inhibits the proliferation of human MHCC97-H liver tumor cells. In the present study, in order to assess the impact of different concentrations of ATO (0, 0.1, 0.2, 0.5, 1, 2, 4, 8 and 10 µM) on the proliferation of MHCC97-H cells, a CCK-8 assay was performed. As shown in Fig. 2A, ATO markedly inhibited the proliferation of MHCC97-H cells at concentrations between 1 and 10 µM in a time- and dose-dependent manner. However, the lower concentrations of ATO (0.1, 0.2 and 0.5 µM) failed to reduce cell viability to an appreciable extent. Therefore, concentrations of 0.1, 0.2 and 0.5 µM were used to further investigate the migration and invasion of the MHCC97-H cells.

ATO reduces the mRNA expression of B7-H4 in human MHCC97-H HCC cells. The mRNA expression of B7-H4 was markedly decreased in the cells treated with 0.1, 0.2 and 0.5 µM ATO compared with that in the control cells (Fig. 2B). Western blotting revealed that the expression levels of B7-H4 were reduced by 11.6, 38.3 and 58.9%
on administration of 0.1, 0.2 and 0.5 µM ATO, respectively (Fig. 2C and D). These results suggested that treatment of the human liver tumor cells with moderate concentrations of ATO effectively suppressed the expression of B7-H4.

**ATO inhibits the migration and invasion of human MHCC97-H HCC cells.** The role of ATO on the migration of MHCC-97-H cells was further examined using a wound healing assays. In the present study, the migration of cells treated with ATO (0.1, 0.2 and 0.5 µM) was markedly decreased in a dose-dependent manner compared with the control group (Fig. 3A and B).

The invasive capability of the MHCC97-H cells treated with or without the different concentrations of ATO was determined using a Transwell assay. The results of the cell invasion assay revealed that ATO at concentrations of 0.1, 0.2 and 0.5 µM markedly inhibited the invasive capability of the cells compared with the control group (Fig. 4A and B).
JAK2/STAT3 signaling is inhibited by ATO. The JAK2/STAT3 signal transduction pathway, as a downstream pathway, is regulated by the expression of various genes. It occupies a central role in cell growth, cell survival, differentiation and oncogenesis. Numerous previous studies demonstrated that the activation of JAKs is switched off by either their autophosphorylation or transphosphorylation by other JAKs associated with cytokine receptors (19). The function of JAKs is closely associated with the activation of the STAT signaling pathway. To determine the effects of ATO on the phosphorylation of JAK2 and STAT3 in MHCC97-H cells, western blot analysis was performed with specific antibodies raised against p-JAK2 and p-STAT3. The results failed to reveal any appreciable difference in the protein expression levels of JAK2 and STAT3 among the different groups. However, a marked decrease in the phosphorylation levels of JAK2 and STAT3 were observed in the cells treated with ATO when compared with those in the control cells (Fig. 5A-C). These results demonstrated that JAK2/STAT3 signaling was inhibited by ATO.

ATO inhibits the expression levels of MMP2 and VEGF. The expression of MMP2, which is considered as an indicator of invasive capability, was further identified by western blotting (20,21). The mRNA expression levels of MMP2 were markedly decreased in the MHCC97-H cells treated with 0.1, 0.2 and 0.5 µM ATO compared with the non-treated group (Fig. 6A). In addition, decreases in the protein expression of MMP2 were also observed in HCC MHCC97-H cells treated with 0.2 and 0.5 µM ATO, as assessed by the western blot analysis (Fig. 6B).

High expression levels of VEGF in HCC is closely associated with tumor neovascularization, growth and metastasis (22,23). The present study revealed that the mRNA and protein expression levels of VEGF in MHCC97-H cells was markedly downregulated following treatment with ATO (0.1, 0.2, 0.5 µM) compared with that in the control cells (Fig. 6A, B and D).

Discussion

B7-H4, a member of the B7 family of transmembrane proteins, was reported to serve as a negative regulator of T cell function (24). The overexpression of B7-H4 promotes cellular transformation, and the B7-H4 protein is not constitutively expressed in healthy peripheral tissues in humans. Several previous studies have suggested that the expression of B7-H4 promotes tumorigenesis in ovarian cancer, renal carcinoma and breast cancer (25-28). Sun et al (28) demonstrated that the downregulation of B7-H4 effectively inhibits the migration and invasion of human non-small cell lung cancer.

ATO (termed ‘Pishuang’ in Chinese) is a commercially valuable arsenic compound, characterized by its acute toxicity. Previous studies revealed that ATO exhibits favorable antitumor activity towards a variety of cancer types, including colon, liver, kidney, bladder, cervix carcinoma and gastric cancer (29). However, the mechanisms by which signal transduction pathways mediating cellular functions are disrupted upon ATO treatment remain to be fully elucidated.

In the present study, it was demonstrated that treatment of the MHCC97-H HCC cells with ATO at concentrations between 1 and 10 µM markedly inhibited cell proliferation, and therefore concentrations of 0.1, 0.2 and 0.5 µM were employed for the migration and invasion studies (Fig. 1). The expression of B7-H4 in ATO-treated liver cancer cells and the control group was subsequently investigated. Compared with the control group, the levels of B7-H4 in the cells treated with 0.1, 0.2 and 0.5 µM ATO were significantly decreased. This result implied that the expression of B7-H4 was markedly deceased upon treatment with ATO.

The influence of ATO on MHCC97-H cell migration and invasion was further assessed. The cellular migration abilities of the MHCC97-H cells treated with ATO were markedly reduced (Fig. 2). In addition, the invasive capability of the cells treated with 0.2 and 0.5 µM ATO was markedly reduced compared with the control group (Fig. 3). The processes of migration and invasion in liver carcinoma are regulated by multiple signaling pathways. The JAK2/STAT3 signal transduction pathway is considered to be an important pathway for the regulation of cell proliferation, differentiation, apoptosis and immune regulation, and therefore, it exerts an essential role in the tumorigenesis and progression of HCC. In the present study, the influence of ATO on JAK2/STAT3 signal transduction was assessed. The results indicated that the phosphorylation levels of JAK2 and STAT3 in ATO-treated HCC cells were markedly lower compared with those in the control cells, suggesting that JAK2/STAT3 signaling in the ATO-treated HCC cells was possibly inhibited by the downregulation of B7-H4.

MMP2 is characterized by its capability to degrade the extracellular matrix, and high expression levels of MMP2 occupy a central role in tumor migration and invasion (30,31). Consequently, the protein level of MMP2, which is correlated with the invasive ability of carcinoma cells, was examined by RT-qPCR and western blotting in the present study. The results demonstrated that the decreased mRNA and protein expression levels of MMP2 were markedly different in the treatment groups compared with the control group. VEGF, previously identified as a potent angiogenic cytokine, which induces mitosis and regulates the permeability of endothelial cells, exerts an essential role in liver tumor angiogenesis and invasion (22). The mRNA and protein expression levels of VEGF in the MHCC97-H cells were also downregulated. These results may account for the reduced migration and invasion capabilities of the ATO-treated HCC cells. ATO treatment also resulted in markedly reduced expression levels of MMP2 and VEGF.

In conclusion, these results suggested that ATO may be a putative compound leading to the further development of anticancer therapeutic agents for the treatment of human liver cancer, since it reduces the expression levels of B7-H4 in MHCC97-H HCC cells, and further affects the tumorigenesis and progression of HCC. These findings provided insights into the molecular mechanisms of liver cancer progression and pathogenesis, and are potentially useful for the development of therapeutic approaches for the disease.

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