The anti-tumor effect of combined treatment with arsenic trioxide and interferon-α on transplanted murine Lewis lung carcinoma

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Abstract : In the present study, we expected the anti-tumor effect by combined treatment of arsenic trioxide and interferon (IFN)-α on murine Lewis lung carcinoma (LL2) cells through in vivo study. As a experimental model, LL2 cells (1×10^6/mouse) were injected subcutaneously into the back region of mice. When the tumor volume reached 100 mm^3, mice were treated with 1 mg/kg arsenic trioxide, 50000 IU IFN-α, or arsenic trioxide and IFN-α. The development of tumor cells was significantly inhibited by combined treatment with arsenic trioxide and IFN-α. In arsenic trioxide and IFN-α treated group, apoptotic index was reached a peak value at 48 hr after the treatment and it was restored to approximately the control level at 8 days. Also, positive signals of Bax and Bad were increased at 48 to 96 hr and decreased at 8 day. Whereas, positive cells of Bcl-2 were steadily decreased at 12 to 48 hr and restored to the background level at 8 days. Our data showed that immunoreactivity of Bcl-2 was decreased at 12 to 48 hr, while positive signals of Bax and Bad were increased in accordance with apoptotic index at these times. In conclusion, our results suggest that the combined treatment with arsenic trioxide and IFN-α significantly inhibited the growth of LL2 tumor cells and induced apoptosis through the up and down-regulation of Bcl-2 gene family.

Key words : arsenic trioxide, IFN-α, apoptosis, Bcl-2, Bax, Bad

Introduction

Arsenic trioxide has been used as a therapeutic agent for the acute promyelocytic leukemia (APL). Especially, it was effective APL patients resistant to all-trans retinoic acid (atRA) or other chemotherapeutic drugs [22]. However, in the majority of patients given long-term, toxic side effects which including skin pigmentation, keratosis, cirrhosis, polyneurites, and gastrointestinal problems were observed. Thus, many researchers have focused on the anti-tumor activity of arsenic trioxide through combination with other types of agents, interferon (IFN)-α, atRA, and chemotherapeutic agents [5, 11, 12, 24, 26, 27]. It was reported that the chronic administration of IFN-α or IFN-β can produce regression of vascular tumors, including Kaposi’s sarcoma, pulmonary hemangiomatosis and hemangiomas [6, 17, 23]. Furthermore, IFN has functions the anti-proliferative effect as well as an immunomodulatory activity in vitro and in vivo [7]. Recently, a high synergistic effect between arsenic trioxide and IFN was reported in human T-cell lymphotropic virus type 1 (HTLV) infected cells [14]. Previous studies explained that arsenic trioxide and IFN combination therapy induced apoptosis and cell cycle arrest.

To inhibit the tumor cellular growth, the processing of apoptosis is an important mechanism [3, 18]. The induction of apoptosis by arsenic trioxide involves inhibition of glutathione peroxidase (GPx) activity and increasing of cellular H_2O_2 content. These are followed...
by cytochrome C release, caspases 3 activation, DNA fragmentation, and the classic morphological changes of apoptosis [10, 13]. Also, arsenic trioxide directly induced apoptosis through the down-regulation of Bcl-2 in NB4 cells of APL [4]. Previous studies suggest that the induction of apoptosis is a critical event for the suppression of tumor growth. Especially, a number of cellular proto-oncogenes including Bcl-2 and its family gene are important in the regulation of apoptosis. Also, Bcl-2 gene family regulated the apoptosis of tumor cells in a various cancer [2, 9]. The levels of Bcl-2 are regulated by closely related Bcl-2 gene family members such as Bax, Bad, Bcl-x(L), and Bcl-x(S). In these genes, Bcl-2 and Bcl-x(L) are known as inhibitor of apoptosis, whereas Bax and Bcl-x(S) are known as inducer of apoptosis [15]. It was reported that change in the ratio of Bcl-2 to Bax expression is the critical determinant of cell fate, cell survival and death. Thus, we suggested the possibility that Bcl-2 gene family, Bcl-2, Bax, and Bad regulated the anti-tumor effect by the combined treatment with arsenic trioxide and IFN-α. However, it has been unknown. The present study was performed to investigate the anti-tumor effect by the combined treatment with arsenic trioxide and IFN-α. The body weight and general physical status of the animals were observed every day.

**Materials and Methods**

**Tumor cell and animal**

The Lewis lung carcinoma cell line which obtained from the American type culture collection (ATCC, Rockville, MD, USA) was cultured in Dulbecco’s modified Eagle medium (DMEM: Gibco, USA) supplemented with 10% heat inactivated fetal bovine serum (Sigma, USA), 100 U/ml penicillin, and 100 U/ml streptomycin (Sigma, USA). The cells were maintained in a humidified atmosphere, at 37°C, 5% CO2 in air. C57BL/6 male mice (110 heads, 7-8 week old), were bred and maintained in the Asan Institute for Life Sciences specific-pathogenic-free (SPF) mouse colony, were housed in wire-bottomed cages in a room with constant temperature (22±1°C) and humidity (55%) and with a 12 hr light (6 a.m. - 6 p.m.) and dark cycle, with free access to food and tap water. Lewis lung carcinoma cells (1x10⁶/mouse) were injected subcutaneously into the back region of mice, tumor volume was measured with a digimatic calipers (Mitutoyo, Japan) at three times a week, and calculated by the formula \( \pi/6 \times w1 \times w2 \times w3 \), where \( w1 \) represented the largest tumor diameter, \( w2 \) represented the smallest tumor diameter and \( w3 \) represented the tumor height. When each tumor volume reached 100 mm³, each animal was treated with 1 mg/kg arsenic trioxide and/or 50000 IU IFN-α. The TUNEL assay

The mice were killed by cervical dislocation at 0, 12, 24, 96 hr and 8 days after drug treatment. Tumor tissues were immediately fixed in 10% neutral buffered formalin solution (pH 7.0), embedded in paraffin and cut into 4 µm thick sections. Sections were deparaffinized in xylene, dehydrated through graded alcohol, and washed 0.1 M phosphate-buffered saline (PBS). Sections were incubated 20 mg/ml proteinase K for 40 min. After washes in PBS, sections were incubated with equilibration buffer followed by TdT enzyme (Oncogene, USA) in a humidified chamber at 37°C for 1 hr, and then a stop/wash buffer was applied for 30 min at 37°C. The sections were incubated with anti-digoxigenin peroxidase (Oncogene, USA) for 30 min at room temperature, counterstained with hematoxylin. With the TUNEL method, five fields of non-necrotic areas were randomly selected in each histological specimen, and the number of apoptotic positive nucleus in each field was calculated as cell numbers per 100 cells.

**Immunohistochemical studies of Bcl-2 gene family**

To determine Bcl-2 gene family, Bcl-2, Bax, and Bad immunoreactivity, immunohistochemistry was performed using an avidin-biotin-peroxidase complex method (Vectastain ABC kit; Vector, USA). Endogenous peroxidase was blocked with a 3% hydrogen peroxide solution for 5 min. The sections were washed in PBS, incubated with normal goat serum to prevent nonspecific binding. Anti-mouse monoclonal Bcl-2, Bax, and Bad antibody (Santa Cruz, USA) were diluted 1:100, 1:200, or 1:100, respectively, were incubated at 4°C overnight. After incubation, the slides were washed in PBS, incubated for 60 min with biotinylated anti-mouse IgG, and then incubated with avidin-biotin-complex according to the manufacturer’s recommendations. After the sections were washed in PBS, the color reaction was performed with 3-amino-9-ethylcarbazole (AEC; Vector, USA). The