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ORIGINAL ARTICLE

Antitumor effects of recombinant human Interleukin-6 on mouse bladder carcinoma through Fas-mediated apoptosis

Chuan-gang Li · Mo-lin Li · Xiao-hong Shu · Yong-ji Liu · Wen-sen Wu

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Abstract
Purpose An apoptosis-inducing therapy is gradually becoming a new strategy for cancer treatment. The aim of this study was to investigate the mechanism of growth-inhibitory effects of recombinant human interleukin-6 (rhIL-6) on bladder tumor-bearing T739 mice in vivo.
Methods Murine bladder transitional carcinoma cells (BTT739) were inoculated subcutaneously into T739 mice as a tumor model for evaluating the antitumor effects of rhIL-6. Then the mice were divided randomly into 5 groups: A, B, C, D and E. Different doses (0, 2, 4, 8×10^6 IU/kg body weight) of rhIL-6 were injected intraperitoneally twice per day and administered for 14 days, and 1 mg/kg/d mitomycin-C(MMC) was used as control. Tumor size was measured and determined as the mean of the largest diameter and the diameter at right angle. Animals were killed by CO_2 inhalation on the 15th day after tumor cell inoculation. Then, tumors were removed, weighed and collected. The tumor growth inhibition rate of rhIL-6 was calculated. The morphological characteristic changes of tumor cells were observed under electron microscope, and cell cycle analysis was determined by flow cytometry. The expressions of Fas, FasL and Bcl-2 protein on tumor cells were qualitatively detected by immunofluorescence cell staining, and their relative contents (rate of positive cells, RPC) were quantitatively determined with flow cytometry.
Results rhIL-6 could inhibit bladder tumor growth in a dose-dependent manner in vivo. The tumor growth-inhibitory rates of 2, 4, 8×10^6 IU/kg rhIL-6 and 1 mg/kg MMC were 11.8, 39.5, 39.7 and 68.8%, respectively. Flow cytometry results showed that a hypodiploid peak before G1 phase could be found in tumor cells treated with rhIL-6. Moreover, the cells treated with rhIL-6 displayed disappearance of nucleoli, chromatin gathering under the nuclear membrane in mass or ring-shape under transmission electron microscopy. The rates of Fas, FasL protein–positive cells estimated by flow cytometry in rhIL-6-treated mice were (12.57 ± 0.83) and (20.1 ± 0.87) %, respectively, significantly higher than that (4.66 ± 0.17) and (14.1 ± 0.83) % in control mice ($P < 0.01$). There was no significant difference in the rate of Bcl-2 protein–positive cells between the mice in these two groups ($P > 0.05$).
Conclusions rhIL-6 had obvious antitumor effects on mouse bladder carcinoma in vivo, and the Fas signaling pathway might play an important role in rhIL-6-induced bladder carcinoma cell apoptosis.

Keywords Recombinant human interleukin-6 (rhIL-6) · Bladder cancer · Fas · Apoptosis · T739 mice

Abbreviations

rhIL-6	Recombinant human interleukin-6
IL-6	Interleukin-6
MMC	Mitomycin-C
BTT739	Murine bladder transitional carcinoma cells
PBS	Phosphate-buffered saline
PI	Propidium Iodide

C. Li · Y. Liu
The Second Affiliated Hospital, Dalian Medical University, 116023 Dalian, People's Republic of China

M. Li (✉) · X. Shu
Dalian Medical University, 116044 Dalian, People's Republic of China
e-mail: molin_li@hotmail.com

W. Wu
The First People's Hospital of Shanghai, 200080 Shanghai, People's Republic of China

i.p.	Intraperitoneally
FADD	Fas-associated death domain
RPC	Rate of positive cells
TEM	Transmission electron microscopy
SPSS	Statistical package for social science
SD	Standard deviation
LSD	Least significant difference
ANOVA	Analysis of variance

Introduction

IL-6 is a multifunctional cytokine produced mainly by T cells, fibroblasts, macrophages and stromal cells in almost all tissues [1], which may act on various kinds of target cells, modulate immune responses and play a fundamental role in inflammatory processes. Mule and his colleagues [2, 3] have demonstrated that recombinant interleukin-6 (rIL-6) could inhibit not only the tumor growth of syngeneic sarcomas (MCA-105, -106 and -203) but also their pulmonary and hepatic metastases in C57BL/6 mice. It was thought that IL-6 had antitumor effects by regulating immune response. Recently, it was reported that IL-6 could induce apoptosis of myeloma tumor cells in vitro via Fas signaling pathway [4–6]. It is known that Fas ligand (FasL) is a type II transmembrane protein expressed particularly on cytotoxic lymphocytes, monocyte macrophages and certain tumor cells [7, 8], and also expressed constitutively in immune privileged sites as, for example, the eye and testis [9]; while its cognate receptor Fas is a type-I membrane receptor protein abundantly expressed by cells in various tissues [10] and particularly on activated T cells, heart cells, kidney cells and hepatocytes. The Fas and FasL system is one of the best-studied cell death systems, and the ligation of Fas by FasL induces the oligomerization of Fas, resulting in the recruitment of Fas-associated death domain (FADD) and the zymogen form of the cysteine protease procaspase 8 to the cytoplasmic tail of Fas [11]. Activation of caspase 8 following Fas–FasL interactions mediates the induction of the proteolytic cascade of apoptosis [12]. It is well known that Fas–FasL pathway is vital for maintaining immune homeostasis. Therefore, IL-6 should be an important cytokine in the host immune and metabolic response to cancer. But little is known about the mechanism of the antitumor effects of IL-6 on the changes of Fas and FasL expression on tumor cells in vivo. The importance of Fas-mediated apoptosis is highlighted in investigating the mechanism of antitumor effects of IL-6. In this study, we examined the inhibitory effects of rhIL-6 on bladder carcinoma T739 mice in vivo in comparison with MMC chemotherapy. Furthermore, the apoptotic effects of rhIL-6 by increasing the expressions of Fas, FasL and bcl-2 protein were also studied. These findings indicate that Fas signaling pathway might play an important role in rhIL-6-induced bladder carcinoma cell apoptosis.

Materials and methods

Reagents

rhIL-6 purified for homogeneity with a specific activity of 1×10^6 U/mg was kindly provided by Professor Xianjun Fan (Chinese Academy of Medical Science, Beijing Union Medical College, PR China). MMC was purchased from Kyowa Hakko Kogyo Co. Ltd (Tokyo, Japan).

Tumor cells

The BTT739 mouse bladder carcinoma model, which was induced by BBN [N-butyl-N-(4-hydroxybutyl nitrosamine)] in the First People's Hospital of Shanghai, PR China, is already established [13]. The tumor tissues excised from the tumor-bearing mice were minced in PBS (phosphate-buffered saline) and then stirred in a triple-enzyme mixture of 0.02 mg/ml deoxyribonuclease type IV, 1 mg/ml collagenase type IV and 0.1 mg/ml hyaluronidase type V (Sigma, USA) for 30 min. The dispersed tumor cells were collected, passed through 100-gauge nylon meshes and washed three times with PBS. The suspensions of tumor cells were adjusted to an appropriate concentration for tumor inoculation.

Mice

Inbred strain mice T739 were purchased from Cancer Institute Animal Center of Chinese Academy of Medical Science and Beijing Union Medical College. The animal studies were conducted in accordance with public Health Service policy and were approved by the local institutional animal care and use committee. All mice used in the experiments were sex- and age-matched and were maintained under specific pathogen-free conditions.

Tumor growth and rhIL-6 treatment in vivo

For tumor establishment, 1×10^6/ml BTT739 cells were washed twice with PBS and injected subcutaneously in a volume of 0.2 ml into the flank of mice. After inoculation, the mice were randomly divided into 5 groups with 10 in each group. Group A: a negative control group, normal saline (NS) was administered in a volume of 0.1 ml/10 g body weight intraperitoneally (i.p.); group B, C and D:

rhIL-6 at a dosage of 2, 4, 8 $\times$ 10^6 IU/kg body weight was administered twice per day i.p., respectively; group E: a positive control group, 1 mg/kg MMC was injected in a volume of 0.1 ml/10 g body weight i.p. Mice were observed for monitoring the signs of drug toxicity subsequently. On the 15th day after inoculation, the mice were killed with carbon dioxide. The tumor tissues were removed and weighed. Inhibition rate of tumor growth after rhIL-6 or MMC treatment was calculated by the following formula: Inhibition rate (%) = [(tumor weight of control group − tumor weight of experimental group)/tumor weight of control group] $\times$ 100%. The tumor tissues were also dissected and collected for further examination.

Cell cycle and apoptosis analysis

Cell cycle distribution in tumor cells after rhIL-6 treatment was determined by staining DNA with propidium iodide (PI, Sigma) as previously described [14]. Briefly, tumor tissues of the mice in group A and group C on the 15th day after inoculation were excised and suspended, respectively. For cell cycle analysis, tumor cell suspensions were prepared and washed with PBS, adjusted to a concentration of 1 $\times$ 10^6 cells/ml and fixed in 70% cold ethanol. The cells were stained in PBS with the addition of 5 mg/ml propidium iodine (PI, Sigma), 1 mg/ml RNase A (Sigma) and 0.1% (v/v) Triton X-100 for 30 min at room temperature in the dark. The cells were analyzed using a FACScan flow cytometer (Becton–Dickinson Immunocytometry System), and the acquired data were analyzed. All experiments were repeated at least in triplicate.

Ultrastructural features of rhIL-6 treated cells with electron microscopic observation

To morphologically demonstrate the induction of tumor cell apoptosis after rhIL-6 treatment in vivo, tumor samples of mice in group A and group C were fixed in 0.1 M ice-cold sodium cacodylate buffer containing 2.5% glutaraldehyde at room temperature for 1 h, postfixed with 1% osmium tetroxide for 1 h and dehydrated through gradient ethanol, then stained with uranyl acetate and lead citrate. The tissue slices were observed under EX-JEM-200 transmission electron microscopy (Japan).

Immunofluorescence cell staining of Fas, FasL and Bcl-2 protein

Fas, FasL or Bcl-2 protein expression on tumor cells was qualitatively detected by immunofluorescence cell staining. On the 15th day after tumor cell inoculation, tumor tissues of mice in group A and group C were excised and suspended. The tumor cell suspensions were incubated with anti-CD95-FITC (B.D., USA), anti-CD95-ligand-FITC (B.D., USA) and anti-Bcl-2 protein-FITC (B.D., USA) in the dark for 1 h at 4°C, respectively. The slides were examined using a fluorescence microscope with appropriate filters. Staining with FITC-conjugated IgG mAb (B.D., USA) was performed as a negative control.

Flow cytometry analysis of Fas, FasL and Bcl-2 protein expression

The relative contents (rate of Fas, FasL or Bcl-2 protein–positive cells,RPC) were quantitatively determined with flow cytometry. On the 15th day after inoculation, tumor tissues from mice in group A and group C were excised and suspended. After fixation with 75% ethanol, cells were washed with PBS, blocked with 0.1% BSA (in PBS) for 10 min at 4°C. The tumor cell suspensions were incubated with anti-CD95-FITC, anti-CD95-ligand-FITC and anti-Bcl-2 protein-FITC in the dark at 4°C for 1 h, respectively, and the isotype-matched FITC-conjugated mouse IgG (B.D., USA) was used as a negative control. The stained cells were analyzed using a FACScan flow cytometer. The data were analyzed by Cellquest software.

Statistical analysis

The data were presented as mean ± SD and were analyzed with SPSS 11.5 statistic software (SPSS Inc, Chicago, IL, USA). Group differences in tumor weight and tumor growth inhibition rate were analyzed by a one-way ANOVA, and post hoc multiple comparison was performed with the LSD method. The rates of Fas, FasL or Bcl-2 protein–positive cells (RPC) were analyzed using a paired Student's t test. For all tests, the significance level for statistical analysis was set at $P < 0.05$.

Results

Evaluation of antitumor effects

After 2 $\times$ 10^5 BTT739 cell inoculation, mice were randomly divided into 5 groups. 2, 4, 8 $\times$ 10^6 IU/kg/d rhIL-6 or 1 mg/kg/d MMC was administered i.p. for 14 days in a volume of 0.1 ml/10 g body weight, respectively, and the same volume of NS was used as control. To evaluate the antitumor effects of rhIL-6 on mouse bladder carcinoma in vivo, tumor tissues from mice in every group were dissected carefully from the surrounding tissues on the 15th day after inoculation and then weighted. The inhibition rates were calculated and are shown in Table 1. The rates of tumor growth inhibition in group B, C, D and E were 11.81, 39.43, 39.72, 68.80%, respectively. These data

Table 1 The inhibitory effects of rhIL-6 on the BTT739 bladder tumor growth in vivo (mean ± SD, $n = 10$)

Groups	Dosage	Tumor weight (g)	Inhibition rate (%)
A (NS)	0.1 ml/10 g	7.80 ± 0.46	–
B (rhIL-6)	2×10^6 IU/kg	6.88 ± 0.46*(a,b)	11.81 ± 0.50*(a,b)
C (rhIL-6)	4×10^6 IU/kg	4.72 ± 0.56*(a,b)	39.43 ± 1.11*(a,b)
D (rhIL-6)	8×10^6 IU/kg	4.70 ± 0.54*(a,b)	39.72 ± 1.44*(a,b)
E (MMC)	1 mg/kg	2.43 ± 0.42*(a)	68.80 ± 1.97*(a)

After 2×10^5 BTT739 cell inoculation, mice were randomly divided into 5 groups. Group A: normal saline (NS) was administered i.p. as a negative control; group B, C and D: different doses of rhIL-6 (2, 4, 8×10^6 IU/kg) were injected intraperitoneally twice per day and were administered for 14 days, respectively; group E, 1 mg/kg/d MMC was administered i.p. for 14 days as a positive control. On the 15th day after inoculation, the mice were skilled and tumors were weighed. The inhibition rates of tumor growth after rhIL-6 or MMC treatment were calculated

* Represents statistical significance ($P < 0.01$); (a) Compared with group A (negative control, $df = 4$, $F = 1588.11$); (b) Compared with group E (positive control; $df = 3$, $F = 2889.35$)

indicated that rhIL-6 could inhibit tumor growth of mice in vivo. The antitumor effects of MMC were significantly greater than that of rhIL-6 ($P < 0.01$), and there was a significant difference in tumor growth inhibition rates between 2, 4, 8×10^6 IU/kg rhIL-6 treated mice and control mice ($P < 0.01$, $P < 0.01$, $P < 0.01$ respectively). Among different doses of rhIL-6 used in this experiment, 4×10^6 IU/kg of rhIL-6 was the minimum dose that had a better antitumor effect in vivo and was used in this study.

Morphological changes

In order to characterize the morphological changes of apoptosis in tumor cells after rhIL-6 treatment in vivo, ultrastructural details were determined using transmission electron microscopy (TEM). As seen in Fig. 1, the chromatin was coagulated beneath the nuclear membrane in mass or ring-shape and the nucleoli were disappeared in tumor cells after rhIL-6 treatment. No typical signs of apoptosis of tumor cells in control mice were detected. These data indicated that rhIL-6 could induce tumor cell apoptosis in vivo.

Effect of rhIL-6 treatment on apoptosis and cell cycle distribution

Flow cytometric analysis of propidium iodide-stained cells is a well-accepted method for quantitative assessment of apoptosis [13]. Cells undergoing apoptosis are identified by the subdiploid (sub-G1) peak on staining with PI. This is attributed to the loss of oligonucleotide size DNA fragments by DNase digestion in the early stages of programmed cell death. And the sub-G1 apoptotic population was calculated by ModiFit LT 2.0 computer program (Verity Software House, Topsham). Flow cytometric analysis of the number of apoptotic cells pre- or post- rhIL-6 treatment was showed in Fig. 2. As seen in Fig. 2, rhIL-6 could induce a significant increase of hypodiploid cells before G1 peak (sub-G1 apoptotic population). The value of sub-G1 apoptotic population was (22.23 ± 1.39)% of tumor cells in rhIL-6 treated mice, while only (5.91 ± 1.84)% of tumor cells in control mice. There was a significant difference between them ($P < 0.01$).

The expression of Fas, FasL and Bcl-2 protein on tumor cells

To verify whether Fas, FasL and Bcl-2 proteins were expressed on tumor cells, immunofluorescence staining was used in this study. Fas and FasL were expressed exclusively in cell membranes, whereas immunoreactivity of bcl-2 was cytoplasmic. The rates of Fas, FasL or Bcl-2 protein–positive cells (RPC) were quantitatively determined with flow cytometry, and the results are shown in Fig. 3. As shown in Fig. 3, the rates of Fas and FasL protein–positive cells were (12.57 ± 0.83) and (20.1 ± 0.87)%, respectively, in tumor cells of mice treated with rhIL-6, and only (4.66 ± 0.17) and (14.1 ± 0.83)% in tumor cells of control mice (Fig. 3). The rate of Fas or FasL protein–positive cells was significantly higher in tumors from rhIL-6-treated mice than from control mice ($P < 0.01$). However, there was no significant difference in the rate of Bcl-2 protein–positive cells between rhIL-6-treated mice and control mice ($P > 0.05$).

Discussion

IL-6 is a cytokine primarily produced by stimulated monocytes, fibroblasts and epithelial cells. It has a wide variety of biological functions through binding to a high-affinity receptor complex consisting of two membrane glycoproteins: an 80-kDa IL-6 binding receptor protein (IL-6R) and a 130-kDa signal transducing protein consisting of a 19 amino acid residue signal peptide (gp130). It was reported that IL-6 might promote the growth of immune cells and enhance the activity of NK cells, LAK cells and lymphocytes infiltrating in the tumor tissue and play a fundamental role in the regulation of the immune response, inflammation and hematopoiesis [15]. It was reported that IL-6 had antiapoptotic activity in multiple myeloma cells [16, 17]. Overproduction of IL-6 also contributes to the development of malignant diseases such as multiple myeloma and renal cancer [15]. But it was also reported that rIL-6 could inhibit the growth of C57BL/6 mice's syngeneic sarcomas (MCA-105, -106, and -203)

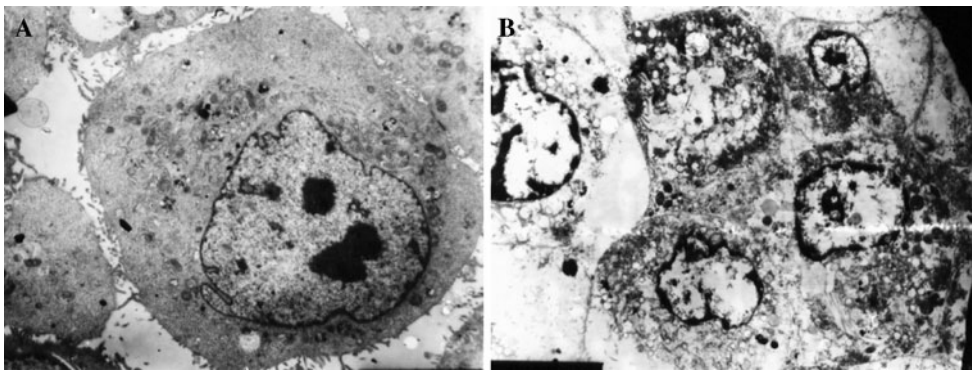

Fig. 1 rhIL-6 induces BTT739 tumor cell apoptosis under electron microscopy observation. **a** The tumor cells from control mice (group A) showed no typical signs of apoptosis. **b** The tumor cells from 4×10^6 IU/kg rhIL-6-treated mice (group C) revealed that the chromatin was coagulated and gathered beneath the nuclear membrane in mass or ring-shape and the nucleoli were disappeared after rhIL-6 treatment

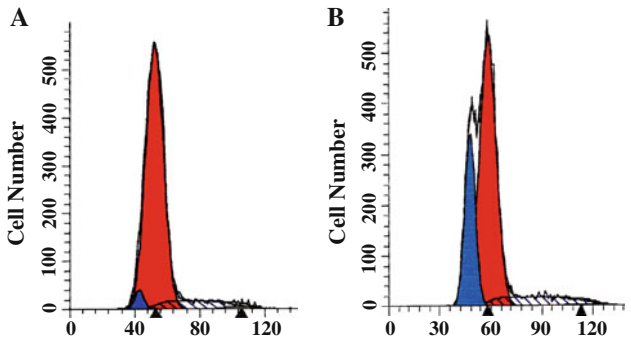

Fig. 2 Flow cytometric analysis on apoptosis of the BTT739 tumor cells by PI-DNA staining. **a** BTT739 tumor cells from control mice (group A); **b** BTT739 tumor cells from rhIL-6-treated mice (group C). A distinct subdiploid *peak* was seen in tumor cells after rhIL-6 treatment. Data are representative of three separate experiments

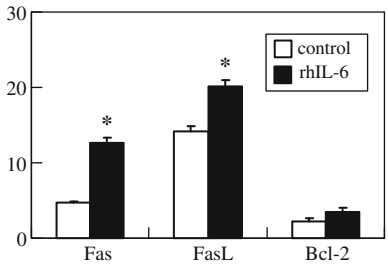

Fig. 3 Comparison of positive rates of Fas, FasL and Bcl-2 protein in tumor cells after rhIL-6 treatment. The expression of Fas, FasL and Bcl-2 protein in BTT739 tumor cells, which extracted from the mice treated with NS (group A) and rhIL-6 (group C), were examined by flow cytometric. The data were presented as mean ± SD. The rates of Fas and FasL protein–positive cells were significantly increased after rhIL-6 treatment at dosage of 4×10^6 IU/kg (*asterisks*), compared with NS group, $P < 0.01$

directly and could inhibit its pulmonary and hepatic metastasis as well [2, 3]. Minami [6] proposed that the stimulus of IL-6 might not only promote growth but also activate the death signal, and PI-3-K might play a key role in the apoptosis signaling pathway of multiple myeloma induced by IL-6. However, the mechanism underlying the antitumor activities of IL-6 is still unclear.

In the present study, we demonstrated that rhIL-6 could not only inhibit mouse BTT739 bladder tumor growth but also induce tumor cell apoptosis in vivo. Tumor weights were lighter in rhIL-6-treated mice than in control mice. Typical signs of apoptosis, such as coagulated chromatin beneath the nuclear membrane in mass or ring-shape, were found by TEM in tumor cells of mice after rhIL-6 treatment. And flow cytometry cell cycle analysis showed a specific hypodiploid peak in tumor cells of mice after rhIL-6 treatment. These data indicated that rhIL-6 could inhibit tumor growth in vivo and could also induce tumor cell apoptosis.

It is known that Fas/FasL system provides a major apoptotic mechanism for many different types of cells, and Fas-mediated apoptosis plays an important role in cytotoxic T-cell-mediated and natural cell-mediated cytotoxicity against cancer cells [18]. When FasL binds to Fas on Fas-sensitive target cells, the target cells will die by apoptosis [19]. The expression of FasL in non-lymphoid tissue is important for protecting immune-privileged sites from immune-mediated damage [9, 20]. It is also known the proteins of the Bcl-2 family possess either pro- or antiapoptotic activity, and the expression of Bcl-2 may inhibit apoptotic cell death induced by various stimuli [21]. One of the most important findings in this study was that the expressions of Fas and FasL protein were up-regulated on bladder tumor cells after rhIL-6 treatment in vivo, but the expression of Bcl-2 protein was not changed after rhIL-6 treatment.

In conclusion, we have demonstrated that rhIL-6 has significant antitumor activities in vivo. It could induce tumor cell apoptosis and up-regulate the expression of Fas and FasL proteins on tumor cells. These data indicated that

a possible mechanism that rhIL-6 inhibited tumor growth in vivo was to induce BTT739 tumor cell apoptosis through Fas signal transduction pathway. These findings may increase our understanding of the mechanisms involved in the induction of apoptosis by rhIL-6 in mouse bladder cancer in vivo. And the mechanisms underlying the Fas-mediated apoptotic signaling pathways controlled by the stimulation of IL-6 and its relationship to bladder tumor cells will be investigated further.

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