
Bcl-2 Induces ROS and Blocks Apoptosis in Bladder Cancer Cells

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RESERACH ARTICLE

Bcl-2 Overexpression Inhibits Generation of Intracellular Reactive Oxygen Species and Blocks Adriamycin-induced Apoptosis in Bladder Cancer Cells

Chui-Ze Kong*, Zhe Zhang

Abstract

Resistance to induction of apoptosis is a major obstacle for bladder cancer treatment. Bcl-2 is thought to be involved in anti-apoptotic signaling. In this study, we investigated the effect of Bcl-2 overexpression on apoptotic resistance and intracellular reactive oxygen species (ROS) generation in bladder cancer cells. A stable Bcl-2 overexpression cell line, BIU87-Bcl-2, was constructed from human bladder cancer cell line BIU87 by transfecting recombinant Bcl-2 [pcDNA3.1(+)-Bcl-2]. The sensitivity of transfected cells to adriamycin (ADR) was assessed by MTT assay. Apoptosis was examined by flow cytometry and acridine orange fluorescence staining. Intracellular ROS was determined using flow cytometry, and the activities of superoxide dismutase (SOD) and catalase (CAT) were also investigated by the xanthinoxidase and visible radiation methods using SOD and CAT detection kits. The susceptibility of BIU87-Bcl-2 cells to ADR treatment was significantly decreased as compared with control BIU87 cells. Enhanced expression of Bcl-2 inhibited intracellular ROS generation following ADR treatment. Moreover, the suppression of SOD and CAT activity induced by ADR treatment was blocked in the BIU87-Bcl-2 case but not in their parental cells. The overexpression of Bcl-2 renders human bladder cancer cells resistant to ADR-induced apoptosis and ROS might act as an important secondary messenger in this process.

Keywords: Bcl-2 - BIU87 - ADR - ROS - apoptosis - SOD - CAT - bladder cancer cells

Introduction

Bladder cancer is the most happened urological tumor in China. The anti-apoptotic mechanisms had been shown to contribute to the drug resistance, consequently leading to post-surgical recurrence, in the malignant tumors. Overcoming apoptotic resistance was therefore supposed to offer a therapeutic benefit for bladder cancer.

B-cell leukemia/lymphoma 2 (Bcl-2) encoded a 26-kDa membrane associated protein that was initially shown to inhibit the cell death induced by IL-3 deprivation (Guthridge et al., 2004). Induction of apoptosis by diverse stimuli, such as radiation, hyperthermia, growth factor withdrawal, glucocorticoids and multiple classes of chemotherapeutic agents, was inhibited by Bcl-2 (Ailawadi et al., 2012; Akurek et al., 2012; Pedersen et al., 2012; Shang et al., 2012; Spampanato et al., 2012). Several models had been proposed to explain the Bcl-2 signaling pathway. Biochemical and genetic evidence indicated that Bcl-2 blocked most forms of apoptosis by preventing mitochondrial changes, such as the release of cytochrome c and an apoptosis-induced factor from the intermembrane space into the cytoplasm (Hasan et al., 2011; Korbakis et al., 2012; Spampanato et al., 2012). Hockenberry et al. (1990) found that overexpression of Bcl-2 decreased the lipid peroxidation and increased the resistance to apoptotic killing by hydrogen peroxide, menadione and the depletion of glutathione in murine lymphocytes, suggesting that the Bcl-2 might make a function as a prooxidant and protect the cells from the oxidative stress by activating the cellular antioxidant defense system.

Oxidative stress is defined as the manifestations of cell or tissue following exposure to excess oxidants. Reactive oxygen species (ROS), such as O$_2^-$, ·OH, and H$_2$O$_2$, are the principal species of intracellular oxidants (Leonard et al., 2010). They are generated as by-products of electron transport through the mitochondrial respiratory chain as well as by γ-ray and ultraviolet light irradiations (Kaur et al., 2001). ROS are highly reactive toward intracellular macromolecules (DNA, proteins and lipids), causing severe lesions that can lead to the cell death by either necrosis or apoptosis, depending on the intensity of the oxidative stimuli (He et al., 2012; Ni et al., 2012; Plante et al., 2012).

There is accumulating evidences that ROS can activate the apoptosis: for example, (1) The addition of ROS or the depletion of endogenous antioxidants can induce apoptosis; (2) The apoptosis can sometimes be inhibited by endogenous or exogenous antioxidants; (3)
The apoptosis was sometimes associated with the increase of intracellular ROS level (Nicolaou-Galmés et al., 2011). Treatments that promoted intracellular ROS formation, for example, with chemicals that inhibited intracellular antioxidant functions or that promoted ROS formation, can also induce apoptosis (Kovacic et al., 2005). In the present study, we found that bladder cancer cells which Bcl-2 was overexpressed in were resistant to adriamycin (ADR)-induced apoptosis. The anti-apoptotic activity of Bcl-2 might be attributed to its capability to sustain the activity of superoxide dismutase (SOD) and catalase (CAT), and to block intracellular ROS production following ADR treatment.

Materials and Methods

Cell lines and tissue culture

Human bladder cancer cells BIU87 were obtained from China Center for Type Culture Collection (Wuhan, China). Cells were cultured in RPMI 1640 (GIBCO, NY) containing 10% fetal calf serum (GIBCO, NY), 100 IU/ml of penicillin (GIBCO, NY) and 100 mg/ml of streptomycin (GIBCO, NY). The cells were cultured at 37°C in a humidified atmosphere with 5% CO2.

Plasmid construction and transfection

A Bcl-2-expressing plasmid [pcDNA3.1(+)–Bcl-2] was prepared using the standard recombinant DNA methods. Briefly, the 847-bp cDNA carrying the entire protein coding sequence of bcl-2 was inserted at the EcoRI and XhoI site of the plasmid pcDNA3.1(+)–neo. The transfection of the expression vector pcDNA3.1(+)–Bcl-2 to the BIU87 cell line was performed by a lipofection method using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). G418 (400 μg/ml)-resistant transformant was obtained from the cell line, and the stable monoclonal transformant expressing the human Bcl-2 were selected by Western blot analysis. BIU87 cell transformant expressing the highest Bcl-2 levels were selected and designated as BIU87-Bcl-2. BIU87 cell line was also transfected with plasmid vector pcDNA3.1(+)–neo (BIU87-neo) carrying a G418 antibiotic resistance gene.

Isolation of mRNA, cDNA Synthesis and PCR

mRNAs were extracted from the different transfectant cells using the Trizol kit following the instruction. The RT reaction was carried out in one cycle at 50°C for 30 minutes; 99°C for 5 minutes; 5°C for 5 minutes using the PrimeScript RT-PCR Kit (TAKARA company, Japan). Aliquots of 2μl of first-strand cDNA was mixed with 20μl of the PCR mixture. The PCR reaction was carried out in three steps as follows: 94°C for 3 minutes (one cycle); 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute (30 cycles); 72°C for 5 minutes (one cycle) using the PrimeScript RT-PCR Kit (TAKARA company, Japan). PCR analysis was performed with primers designed specific for Bcl-2 and the product was analyzed by electrophoresis on 1% agarose gel. DNAs were visualized by ethidium bromide staining. The density of the bands was assessed and the relative amount of mRNA of Bcl-2 gene was determined as a ratio to β-actin. The sequences of the primers were designed by the software of Primer 5.0, and synthesized by Sangon company (Shanghai, China). Bcl-2 primers: forward primer 5′-CCAATATGACTCCTGCTG-3′ and reverse primer 5′-TGATGCCCTGGGTAACTCTA-3′, designed for 106 bp. β-actin primers: Forward primer 5′-ACTTAGTTGCGTACCCCT-3′ and reverse primer 5′-TGAACTTTGGGGATGCTCGCTCCA-3′, designed for 190 bp.

Western blot analysis

Western blotting was conducted as previously described (Zhang et al., 2011). Aliquots of total protein extracts from the different transfectant cells were obtained in 0.1 M Tris-HCl buffer, pH 7.0, containing 1% sodium dodecyl sulphate (SDS), 0.05% β-mercaptoethanol, 2.5% glycerol, 0.001% bromophenol blue, boiled for 5 minutes and subsequently size fractionated by 12% SDS-PAGE and then transferred to PVDF blotting membranes. Immunoblots were performed using mouse monoclonal primary antibodies specific for Bcl-2 (Santa Cruz Biotechnology, CA) and mouse monoclonal antibody for β-actin (Abcam, Hongkong, a housekeeping protein used as a loading control to assure equal amounts of protein in all lanes). After blocking nonspecific binding with 5% BSA in TBS (pH 7.5) containing 0.05% Tween-20 (TBST), primary antibodies were incubated on the membranes for Bcl-2 (1:1000) and β-actin (1:2000) overnight at 4°C in TBST. Following three times washes in TBST, the membranes were incubated for 2 hours at 37°C with goat polyclonal secondary antibody to mouse IgG (1:5000, Abcam, Hongkong) labeled with horseradish peroxidase. The proteins were detected using an ECL detection system (Pierce, Rockford, IL), as directed by the manufacturer. Specific bands for Bcl-2 and β-actin were identified by prestained protein molecular weight marker (MBI Fermentas, USA). The EC3 Imaging System (UVP Inc.) was used to catch up the specific bands, and the optical density of each band was measured using Image J software. The ratio between the optical density of interest proteins and β-actin of the same sample was calculated as relative content and expressed graphically.

MTT assay

BIU87, BIU87-neo and BIU87-Bcl-2 cells were suspended at a concentration of 1.0×10⁵ cells per ml in medium, and 0.1 ml of the cell suspension was dispensed into 96-well plates. After 24 hours, the cells were treated with ADR (Pharmacia Chemical Co., Italy) at concentrations of 0, 6.25, 12.5, 25, 50 or 100 μg/ml for 24 hours. The cells were incubated with 10 μl of 5 mg/ml MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] reagent per well for 4 hours and then were dissolved in 100 μl dimethyl sulphoxide. Finally, the optical density value (A) of each well was measured at a measurement wavelength of 490 nm using a plate reader (Model 680, BIO-RAD, UK). Cell growth inhibition ratio was calculated as (1 – A490 of experimental well/A490 of blank control well) × 100%. Each assay was repeated at least 3 times.
Detection of intracellular ROS

BIU87, BIU87-neo and BIU87-Bcl-2 cells (5×10⁴) were cultured in 100 μl culture flask for 48 hours and then were treated with ADR at concentrations of 0, 6.25, 12.5 or 25 μg/ml for 24 hours. The cells were then harvested, washed twice with PBS, and incubated in 2 ml of DCFH-DA (5 μmol/L), agitated 60 minutes at 37°C, resuspended with PBS. The cellular fluorescence intensity was measured in a FACScan cytometry (Becton Dickinson, San Jose, CA). For each analysis, 10000 events were recorded.

Figure 1. Expression of Bcl-2 by RT-PCR AND Western Blot in BIU87, BIU87-neo and BIU87-Bcl-2 Cells. (A) RT-PCR was used to examine the mRNA level of Bcl-2 indicated by band intensities. Line 1: BIU87 cells group; Line 2: BIU87-neo group; Line 3: BIU87-Bcl-2 group. β-actin was used as a loading control to assure equal amounts of protein in all lanes. (B) The ratio between the optical density of Bcl-2 and β-actin of the same group was calculated and expressed graphically. (C) Western blot was used to examine the protein expression of Bcl-2 indicated by band intensities. Line 1: BIU87 cells group; Line 2: BIU87-neo group; Line 3: BIU87-Bcl-2 group. β-actin was used as a loading control to assure equal amounts of protein in all lanes. (D) The ratio between the optical density of Bcl-2 and β-actin of the same group was calculated and expressed graphically. Significant differences were analyzed statistically. The data are representative of three individual experiments. (TIF)

Table 1. The Cell Viability Inhibition of BIU87, BIU87-neo and BIU87-Bcl-2 Cells Following Different Concentration of ADR Treatment for 24 Hours

<table>
<thead>
<tr>
<th>Group</th>
<th>The concentration of ADR(μg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>BIU87</td>
<td>0±0</td>
</tr>
<tr>
<td>BIU87-neo</td>
<td>0±0</td>
</tr>
<tr>
<td>BIU87-Bcl-2</td>
<td>0±0</td>
</tr>
</tbody>
</table>

*There were significant differences (P<0.05) in BIU87-Bcl-2 group compared with in BIU87 group and in BIU-neo group.

Detection of intracellular SOD and CAT

BIU87, BIU87-neo and BIU87-Bcl-2 cells (5×10⁴) were cultured in 100 μl culture flask for 48 hours and then were treated with ADR at concentrations of 0, 6.25, 12.5 or 25 μg/ml for 24 hours. The cells (2×10⁶) were then harvested, washed twice with PBS, added ice-cooled PBS 500 μl, shattered with ultrasonic disintegrator, centrifuged at 3000 rpm, 4°C for 15 minutes. Supernatant protein concentration was determined by Coomassie Brilliant Blue. The activity of SOD and CAT was detected by Xanthinoxidase method and visible radiation method using SOD and CAT detecting kit, respectively, according to the manufacture’s introduction.

Statistical analysis

The T Test was used to evaluate the difference of the Bcl-2 expression in every group. The P Test was used to evaluate the difference in different cells treated with ADR. P values<0.05 were considered statistically significant.

Results

Expression of Bcl-2 in BIU87, BIU87-neo and BIU87-Bcl-2

In order to detect Bcl-2 protein expression in BIU87, BIU87-neo and BIU87-Bcl-2 cells, proteins were taken from the cells. Western blot analysis showed that higher level of Bcl-2 protein was expressed in BIU87-Bcl-2 cells than those in BIU87, BIU87-neo cells, but there was no obviously different in BIU87 compared with in BIU87-neo cells (Figure 1A and 1B). Furthermore, RT-PCR was used to examine the mRNA levels in BIU87, BIU87-neo and BIU87-Bcl-2 cells. The result suggested that the mRNA levels of Bcl-2 in BIU87-Bcl-2 cells than those in BIU87, BIU87-neo cells, but there was no obviously different in BIU87 compared with in BIU87-neo cells (Figure 1C and 1D). These results were compared to the quantity of β-actin, whose expression remained constant in BIU87 cells of every group.
Bcl-2 decreased the sensitivity of BIU87 cells to ADR

To explore the effect of Bcl-2 overexpression on apoptotic resistance, we examined the cell viability inhibition of BIU87, BIU87-neo and BIU87-Bcl-2 cells following ADR treatment for 24 hours. Although BIU87 and BIU87-neo cells expressed a substantial amount of Bcl-2, the cell viabilities were both inhibited after the treatment with ADR. The cytotoxicity of ADR was dose-dependent. On the other hand, the sensitivity of BIU87-Bcl-2 cells to ADR treatment was significantly lower than BIU87 and BIU87-neo cells (Table 1). This result suggested that Bcl-2 overexpression decreased the susceptibility of BIU87 cells to ADR.

Bcl-2 inhibited ADR-mediated apoptosis

Flow cytometric analysis was used to assess apoptosis, which showed that a dose-dependent sub-G1 apoptotic cell population was presented at 24 hours following ADR treatment in BIU87 and BIU87-neo cells. However, the apoptotic cell population in BIU87-Bcl-2 cells after treatment was significantly lower than those in their parental cells (Figure 2A, 2B).

Bcl-2 inhibited ADR-induced intracellular reactive oxygen species

To investigate whether Bcl-2 mediating ADR resistance was involved in scavenging free radicals, we used DCFH-DA, an oxidation-sensitive fluorescent probe, to examine the generation of reactive oxygen species in ADR-treated BIU87, BIU87-neo and BIU87-Bcl-2 cells following different concentrations of ADR treatment for 24 hours. The oxidative stress level was analyzed by flow cytometry in every group. The intracellular reactive oxygen species level was examined by flow cytometry in every group (Figure 3).

Table 2. The Activity of SOD was Examined in BIU87, BIU87-neo and BIU87-Bcl-2 Cells Following Different Concentration of ADR Treatment for 24 Hours (U/mg prot, Mean±SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>The concentration of ADR (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>BIU</td>
<td>98.3±5.44</td>
</tr>
<tr>
<td>BIU87-neo</td>
<td>107.5±6.38*</td>
</tr>
<tr>
<td>BIU87-Bcl-2</td>
<td>126.18±4.93#</td>
</tr>
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</table>

*There were significant differences ($P < 0.05$) compared with in BIU87 group; **There were significant differences ($P < 0.05$) compared with in BIU-neo group.
cells. When BIU87, BIU87-neo and BIU87-Bcl-2 cells were treated with ADR for 24h, DCFH fluorescent intensity was increased with the concentration of ADR. This increase was, however, significantly impaired in BIU87-Bcl-2 cells, suggesting that Bcl-2 blocked the ADR inducing intracellular peroxide production (Figure 3A, 3B).

Effect of ADR on SOD and CAT activity in BIU87 Cells

Since SOD and CAT had been shown as peroxide-scavenging enzymes in bladder cancer cells, the activity of SOD and CAT in three cells following ADR treatment were investigated. With the increase of ADR concentration, the activity of SOD and CAT were significantly decreased in BIU87, BIU87-neo cells, but only slightly reduced in BIU87-Bcl-2 (Figure 4A, 4B, Table 2 and Table 3). This result suggested that Bcl-2 might block ADR-induced SOD and CAT activity reduction.

Discussion

Bcl-2 has been reported to counteract apoptotic cell death through multiple mechanisms, which mainly targets mitochondrial events. Nonetheless, the attention had been recently focused on its role in maintaining or augmenting cellular antioxidant defense capacity that involve antioxidant enzymes (e.g. CAT, SOD, GPx, and GR) and an antioxidant molecule GSH. In the present study, we found that Bcl-2 overexpression rendered the drug resistance in bladder cancer cells. Furthermore, enhanced expression of Bcl-2 in BIU87 cells blocked ADR-induced ROS generation and SOD and CAT suppression. These findings suggested that Bcl-2-mediated drug resistance might be involved in interfering ROS generation.

Many chemotherapeutic drugs were reported to induce apoptosis in cancer cells. In this study, we found that ADR could cause the apoptosis of BIU87 cells. The proto-oncogene Bcl-2 prevented the apoptotic cell death induced by various treatments, including chemotherapy (Newton et al., 2000; Nam et al., 2007; Hasima et al., 2010). Takahashi et al. (Smith et al., 2011; Takahashi et al., 2012) found that although Bcl-2 transfectants were resistant to the drug-induced apoptosis in liver cancer cells, Bcl-2 overexpression did not affect the doxorubicin-induced growth suppression. To examine the anti-apoptotic effect of the Bcl-2 protein, we overexpressed the Bcl-2 gene in human bladder cancer BIU87 cell line. When high levels of Bcl-2 were expressed in BIU87 cells, we found that the cells became resistant to the ADR-induced cellular apoptosis.

As anthracycline derivative, ADR was a highly effective anticancer drug that was widely used in treatment of a broad spectrum of cancers (Liao et al., 2005). One of the proposed mechanisms of ADR-induced apoptosis was an oxidation of cellular components via formation of reactive oxygen species (ROS) (Savatier et al., 2012). Several mechanisms of ADR-mediated ROS generation had been proposed. The quinine moiety of ADR is prone to the generation of oxygen radicals through enzymatic mechanism utilizing mitochondrial respiratory chain and nonenzymatic pathway, which incorporated iron (Berthiaume et al., 2007; Ohyama et al., 2010). In this study, we clearly showed that ADR induced ROS accumulation in bladder cancer cells and the mechanism appeared to involve the suppression of SOD and CAT activities.

The localization of Bcl-2 at the site of oxygen free radical generation, and evidence that ROS were able to cause apoptosis in various cell lines raised the possibility that Bcl-2 prevented the apoptosis by either acting as an antioxidant or by inhibiting the production of free radicals. Evidences from in vitro and in vivo studies suggested that Bcl-2 might block the apoptosis through the regulation of cellular antioxidant defense mechanisms and, in this context, had been considered to act as a free radical scavenger (Andoh et al., 2000).

Table 3. The Activity of CAT was Examined in BIU87, BIU87-neo and BIU87-Bcl-2 Cells Following Different Concentration of ADR Treatment for 24 Hours (U/mg prot, Mean±SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>The concentration of ADR (μg/ml)</th>
<th>0</th>
<th>6.25</th>
<th>12.5</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIU</td>
<td></td>
<td>22.63±4.21</td>
<td>17.22±5.28</td>
<td>13.52±4.85</td>
<td>8.64±3.96</td>
</tr>
<tr>
<td>BIU87-neo</td>
<td></td>
<td>24.27±2.39</td>
<td>19.63±3.83</td>
<td>14.93±2.34</td>
<td>10.96±2.48</td>
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<tr>
<td>BIU87-Bcl-2</td>
<td></td>
<td>39.23±1.61</td>
<td>33.72±1.20*</td>
<td>27.06±0.81*</td>
<td>20.43±2.38*</td>
</tr>
</tbody>
</table>

*There were significant differences (P< 0.05) compared with in BIU87 group; # There were significant differences (P< 0.05) compared with in BIU-neo group

Figure 4. The Activities of SOC and CAT were Examined in BIU87, BIU87-neo and BIU87-Bcl-2 Cells Following Different Concentration of ADR Treatment for 24 Hours. (A) Line graph was used to show the activities of SOC in every group. (B) Line graph was used to show the activities of CAT in every group. (TIF)
instance, the levels of hydroxyl radicals generated by quinoneproducing agents and 3-nitropropionic acid were lowered in Bcl-2-overexpressing cells compared with the vector-transfected control cells (Luanpitpong et al., 2011; Yin et al., 2012). In this study, we found a lower level of ROS was induced by ADR in BIU87-Bcl-2 than those in BIU87 and BIU87-neo cells. This result suggested that Bcl-2 blocked ADR-induced apoptosis probably through decreasing the intracellular ROS level.

Bcl-2-overexpressing cells had been shown to express relatively high levels of antioxidant enzymes and GSH. However, several lines of evidence suggested that the effects of Bcl-2 on the expression or activity of antioxidant enzymes were cell type-specific. For instance, Bcl-2 increased CAT activities in rat pheochromocytoma (PC12) cells but not in the hypothalamic GnRH cell line GT1–7 (Ferreiro et al., 2007). The activities of GPx and GR in Bcl-2-overexpressing PC12 and GT1–7 cells were similar to those of respective control transfectants. Astrocytes overexpressing Bcl-2 exhibited the elevated SOD and GPx activities, but murine lymphoid hematopoietic FL5–12 cells showed no changes in any antioxidant enzyme activity when transfected with Bcl-2 (Brecht et al., 2005). Bcl-2-transfected teratocarcinoma NT-2/D1 and neuroblastoma SK-N-MC cells displayed an increased CuZn-SOD activity, but not those of Mn-SOD, GPx, and GR (Radhiga et al., 2012). In the present study, we found that BIU87-Bcl-2 displayed an increased SOD and CAT activity compared with its parental cells, suggesting that Bcl-2 might inhibit ADR-induced intracellular ROS increasing through the enhancement of both SOD and CAT activities in bladder cancer cells. This observation implied that the putative up-regulation of antioxidative enzymes by Bcl-2 might be crucial for the antiapoptotic activity of Bcl-2 in human bladder carcinoma cells.

In conclusion, we demonstrated that Bcl-2 overexpression inhibited ADR-induced ROS generation in bladder cancer cells, and rendered drug resistance. Understanding of cellular and molecular regulatory mechanisms underling the antiapoptotic functions of Bcl-2 might provide a new antioxidant therapeutic strategy for overcoming drug resistance related to Bcl-2 overexpression.

Acknowledgements

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