RESEARCH ARTICLE

Effect of Bcl-2 on Apoptosis and Transcription Factor NF-κB Activation Induced by Adriamycin in Bladder Carcinoma BIU87 Cells

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Abstract

Resistance to apoptosis is a major obstacle preventing effective therapy for malignancies. Bcl-2 plays a significant role in inhibiting apoptosis. We reconstructed a stable human Bcl-2 transfected cell line, BIU87-Bcl-2, that was derived from the transfection of human bladder carcinoma cell line BIU87 with a plasmid vector containing recombinant Bcl-2 [pcDNA3.1(+)-Bcl-2]. A cell line transfected with the plasmid alone [pcDNA3.1(+)-neo] was also established as a control. BIU87 and BIU87-neo proved sensitive to adriamycin induced apoptosis, while BIU87-Bcl-2 was more resistant. In view of the growing evidence that NF-κB may play an important role in regulating apoptosis, we determined whether Bcl-2 could modulate the activity of NF-κB in bladder carcinoma cells. Stimulation of BIU87, BIU87-neo and BIU87-Bcl-2 with ADR resulted in an increase expression of NF-κB (p<0.001). The expression of NF-κB in BIU87-Bcl-2 was higher than in the other two cases, with a concomitant reduction in the IκBα protein level. These results suggest that the overexpression of Bcl-2 renders human bladder carcinoma cells resistant to adriamycin -induced cytotoxicity and there is a link between Bcl-2 and the NF-κB signaling pathway in the suppression of apoptosis.

Keywords: Bladder cancer - BCL-2 - NF-Kb - apoptosis - adriamycin

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Introduction

NF-κB was discovered by Sen and Baltimore in 1986 as a factor in the nucleus of B cells that binds to the enhancer of the kappa light chain of immunoglobulin (Sen et al., 1986). It has since been shown to be expressed ubiquitously in the cytoplasm of all cell types, from Drosophila to man. It translocates to the nucleus only when activated, where it regulates the expression of over 200 immune, growth, and inflammation genes. Most carcinogens and tumor promoters activate NF-κB, whereas chemopreventive agents suppress it, suggesting a strong linkage with cancer (Jana et al., 2012). Paradoxically most agents, including cytokines, chemotherapeutic agents, and radiation, that induce apoptosis also activate NF-κB (Wang et al., 2012), indicating that NF-κB is a part of the cells’ autodefense mechanism and thus may mediate desensitization, chemoresistance, and radioresistance (Aggarwal et al., 2011).

NF-κB belongs to a family of transcription factors with Rel homology and include Rel A (also called p65), Rel B, c-Rel, p50 (also called NF-κB1), and p52 (also called NF-κB2). Similarly, a family of anchorin-domain containing proteins—IκBα, IκBβ, IκBγ, IκBαε, bcl-3, p105, and p100—keep NF-κB in its inactive state within the cytoplasm. In the cytoplasm, the predominant form of NF-κB consists of a heterotrimer of p50, p65, and IκBα. The phosphorylation, ubiquitination, and degradation of IκBα releases the p50-p65 heterodimer, which then translocates to the nucleus and binds its specific 10 base pair consensus site GGGPuNNPyPyCC (Fenollar-Ferrer et al., 2012).

NF-κB is constitutively active in most tumor cell lines, whether derived from hematopoietic tumors or solid tumors (Fang et al., 2011; Yang et al., 2012). Suppression of NF-κB in these tumor samples inhibits proliferation, causes cell cycle arrest, and leads to apoptosis (Hu et al., 2012), indicating the crucial role of NF-κB in cell proliferation and survival. What causes the constitutive activation of NF-κB in tumor cells is incompletely understood. Mutation of IκBα, enhanced proteosomal activity (Hoehrainer et al., 2012), or enhanced inflammatory cytokine expression have all been cited (Harikumar et al., 2009).

Treatment of cells with various NF-κB-inducing agents results in phosphorylation of IκB or p105 (Kar et al., 2011). Anthracenyl anticaner drugs, such as adriamycin were reported to be potent activators of NF-κB (Sims et al., 2013). Adriamycin allows rapid nuclear translocation of NF-κB through degradation of IκB-inhibitory cytoplasmic retention proteins (Lemmon et al., 2011).

Although the mechanisms that govern apoptosis in

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bladder carcinoma cells remain poorly defined, there is evidence that the bcl-2 gene product may play a critical role in this process. The Bcl-2 gene encodes a 26-kDa membrane associated protein that was initially shown to inhibit cell death induced by IL-3 deprivation (Calviño et al., 2011) and various other stimuli, including chemotherapeutic agents and heat shock (Shelton et al., 2009) biochemical and genetic evidence indicates that Bcl-2 blocked most forms of apoptosis by preventing mitochondrial changes, such as the release of cytochrome c and an apoptosis-inducing factor from the intermembrane space into the cytoplasm (Choi et al., 2012; Werneburg et al., 2012). Since NF-κB has been suggested to play a benificial role in preventing apoptosis provoked under certain conditions, we ascertained whether Bcl-2 modulates the activity of NF-κB in bladder carcinoma cells.

Materials and Methods

Cell lines and culture conditions

BIU87 cells were cultured in RPMI 1640 (GIBCO, NY, USA) containing 10% fetal calf serum (GIBCO, NY, USA), 100 IU/ml of penicillin (GIBCO, NY, USA) and 100 mg/ml of streptomycin (GIBCO, NY, USA). The cells were cultured at 37 °C in a humidified atmosphere with 5% CO2. The medium was changed two to three times a week. Transfectant cells were cultured in the same manner.

Establishment of stable clones expressing human Bcl-2 by plasmid 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 EcoR I 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, MA, USA). G418 (400 μg/ml)-resistant transformant was obtained from the cell line, and 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 carrying a G418 antibiotic resistance gene (neomycin), BIU87-neo.

Detection of Bcl-2 by Western blot analysis

Western blot was carried out as previously described (Zhang et al., 2012). Aliquots of total protein extracts from the different transfectant cells were suspended 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 3 min and subsequently size fractionated by 12% SDS-PAGE. The gel was electroblotted 2 hours onto nitrocellulose paper at 50 V and the bands revealed by a monoclonal anti-human Bcl-2 antibody and anti-human GAPDH antibody (Santa Cruz, USA) and a horse radish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Cambridge, MA) as the secondary antibody. The proteins were detected using an ECL detection system (Pierce, Rockford, IL), as directed by the manufacturer. The EC3 Imaging System (UVI Inc., PA, USA) was used to catch up the specific bands, and the optical density of each band was measured using Image J software.

Cell viability assay

BIU87, BIU87-neo and BIU87-Bcl-2 cells were suspended at a concentration of 1x10^6 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 adriamycin (Pharmacia Chemical Co., Italian) at concentrations of 0, 6.25, 12.5, 25, 50 or 100 μg/ml for 24 hours. The cells were incubated with 10μl of 5mg/ml MTT [3-[4, 5-dimethylthiazolyl-2]-2, 5-diphenyltetrazolium bromide] reagent per well for 4 hours and then were dissolved in 100μl dimethyl sulfoxide. The cell viability could be quantified by spectrophotometric means.

Detection of apoptosis

BIU87, BIU87-neo and BIU87-Bcl-2 cells (5x10^5) were cultured in culture flask for 48 hours and then were treated with adriamycin at concentrations of 0, 6.25, 12.5 or 25 μg/ml for 24 hours. The cells were then harvested, washed with phosphate buffered saline (PBS), fixed in 1ml of iced 70% ethanol at 4°C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 3 ml of PBS. Next, 500μl of propidium iodide solution (containing 5% propidium iodide, 2% RNase, 1% Triton X-100, 100% natrium citricum) was added and incubated at 4°C for 30 min. Fluorescence emitted from the propidium iodide-DNA complex was quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton Dickinson, San Jose, CA).

Detection of NF-κB activation

BIU87, BIU87-neo and BIU87-Bcl-2 cells (5x10^5) were cultured in culture flask for 48 hours and then were treated with adriamycin at concentrations of 12.5 μg/ml for 24 hours. 5x10^5 cells, treated or not, were washed twice in cold PBS and centrifuged (2 min, 4000 rpm, 4°C). The pellet was resuspended in 250μl of hypotonic buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 0.2 mM PMSF, aprotinin 1 μg/ml) and centrifuged (2 min, 4000 rpm, 4°C). Then, pelleted cells were resuspended in 300 μl of hypotonic buffer and incubated on ice for 15 min, 40 μl of NP-40 solution was added, and the cells were vigorously mixed for 10 s and centrifuged (30 s, 15,000 rpm, RT). The cytosolic supernatants were harvested and stored at -80°C in aliquots for later use. Pelleted nuclei were suspended in 40μl of hypotonic buffer B (10 mM Hepes, pH 7.9, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 10% (v/v) glycerol, aprotinin 1 μg/ml), incubated for 30 min on ice, mixed for 10 s, and centrifuged for 10 min at 15,000 rpm at 4°C. The supernatants that contained nuclear proteins were harvested and stored at -80°C in aliquots for later use. Protein concentrations were determined with phenol reagent method. Cells extracts (30 μg) were resolved by electrophoresis in a 10%
**Statistical analysis**

Results are representative of three separate experiments. One-way ANOVA with a Neuman–Keuls post hoc comparison was used for statistical significance, with $p < 0.05$.

**Results**

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

After transfection of BIU87 cells with pcDNA3.1(+) Bcl-2, we selected a stable monoclonal Bcl-2 transfectant from the G418-resistant cells by Western blotting using a highly specific anti-human Bcl-2 antibody and established the monoclonal cell line BIU87-Bcl-2. Western blot analysis of Bcl-2 expression in the BIU87 (parent), control vector (pcDNA3.1(+) neo), and Bcl-2 vector (pcDNA3.1(+) Bcl-2) transfected cells is shown in Figure 1 and confirmed very high level expression of the protein in the latter. Western blot analysis also revealed that endogenous Bcl-2 was expressed in BIU87 cells, and in BIU87-neo cells, the Bcl-2 expression was similar to that in BIU87 cells.

**Effect of adriamycin treatment on the viability of the transfected cell lines**

To examine whether Bcl-2 over-expression decreases sensibility of adriamycin on BIU87, we examined the viability rate of BIU87, BIU87-neo and BIU87-Bcl-2 cell line after adriamycin treatment for 24 hours. Although BIU87 and BIU87-neo cells expressed a substantial amount of Bcl-2, the viability of them all decreased after the treatment with 6.25, 12.5, 25, 50 or 100 μg/ml of adriamycin for 24 hours; and the degree of decrease increased with the raising of the concentration of adriamycin. The similar result could be seen on BIU87-Bcl-2 cell line. The viability rate of BIU87-Bcl-2 cell line after adriamycin treatment was higher than BIU87 and BIU87-neo cell line, and there is no significant difference between the latter two cell line (Figure 2). The results indicated that Bcl-2 over-expression can decrease sensibility of on BIU87 to adriamycin.

**Bcl-2 inhibits adriamycin-mediated apoptosis**

To investigate the effect of Bcl-2 on adriamycin-mediated apoptosis, BIU87, BIU87-neo and BIU87-Bcl-2 cell lines were treated with adriamycin at concentrations of 0, 6.25, 12.5 or 25 μg/ml for 24 hours. Two established criteria were subsequently used to assess apoptosis in our system. Apoptosis in the three cell line was determined using flow cytometric analysis to detect hypodiploid cell populations. Flow cytometric determination of the DNA content of control and adriamycin treated propidium iodide (PI)-stained BIU87, BIU87-neo and BIU87-Bcl-2 cells. The results show that with the raising of the concentration of adriamycin the apoptosis rates were added in the three cell lines. When be treated by the same concentration of adriamycin, the apoptosis rate of BIU87-Bcl-2 cell line was lower than the other two ones (Figure 3). The results indicated that Bcl-2 over-expression can...
inhbit adriamycin-induced BIU87 apoptosis.

Bcl-2 can enhance NF-κB activation

Since degradation of the inhibitory subunit IκBα is a common key event in the activation of NF-κB by diverse stimuli, the effect of adriamycin on IκBα levels in BIU87, BIU87-neo and BIU87-Bcl-2 cell lines was examined. Protein extracts of the three cells were subjected to Western blot analysis and probed with a rabbit antibody directed toward IκBα/MAD-3. As shown in Figure 4, IκBα level was suppressed slightly but not significantly in BIU87-Bcl-2 cells compared with the other two ones. After be treated with 12.5 μg/ml of adriamycin, IκBα levels were all decreased in the three cells, and IκBα levels were profoundly suppressed in BIU87-Bcl-2 cells expressing Bcl-2 compared with the other two cells (Figure 5A). These data suggest that in these BIU87 cells, the NF-κB activation by can be explained by IκBα degradation (Figure 5B). Interestingly this effect was more obvious in BIU87-Bcl-2 cells indicated Bcl-2 can enhance the activation of NF-κB.

Discussion

In this paper, we investigated whether Bcl-2 overexpression affects the sensitivity of the BIU87 cell line to adriamycin. The results obtained using Bcl-2-overexpressing clone derived by transfecting Bcl-2 cDNA to the BIU87 human bladder cancer cell line, expressing the Bcl-2 protein, demonstrated that Bcl-2 overexpression played a key role in modulating drug sensitivity. When high level of Bcl-2 was expressed in BIU87 cells, we found that the cell line became resistant to the adriamycin-induced cellular toxicity. However, BIU87 and BIU87-neo cells remained sensitive to adriamycin although Bcl-2 expression was observed in them. These findings suggested that Bcl-2 expression level was important for the prevention of doxorubicin-induced apoptosis in BIU87 cell lines.

Many chemotherapeutic drugs are reported to induce apoptosis in cancer cells (Nam et al., 2007; Mulvey et al., 2011). In our study, we demonstrated that adriamycin could cause BIU87 cells apoptosis, which confirmed by PI staining. We also found that with the raising of adriamycin concentration (raising from 6.25 μg/ml to 25μg/ml), the apoptosis rates of BIU87, BIU87-neo and BIU87-Bcl-2 were raising. The proto-oncogene Bcl-2 prevents apoptotic cell death induced by various treatments, including chemotherapy (Kelkel et al., 2012; Otterson et al., 2012). Takahashi et al. (2003) had examined the role of Bcl-2 on doxorubicin-induced apoptosis of human liver cancer cells; they found that although Bcl-2 transfectants were resistant to the drug-induced apoptosis, Bcl-2 overexpression did not affect doxorubicin-induced growth suppression. These results suggested that the overexpression of Bcl-2 rendered human BIU87 cells resistant to adriamycin-induced cytotoxicity. To examine this anti-apoptotic effect of the Bcl-2 protein, we over-expressed the human bcl-2 gene in human bladder carcinoma cell line BIU87. When high level of Bcl-2 was expressed in BIU87 cells, we found that the cell line became resistant to the adriamycin-induced cellular apoptosis.

Recently, increasing evidence supports the role of NF-κB in regulation of anti-apoptotic gene expression and promotion of cell survival (Oh et al., 2012). Since that Bcl-2 has been shown to prevent apoptotic cell death in BIU87 cells, we ascertained whether Bcl-2 might enhance the activation of NF-κB. Western blot analysis of protein extract prepared from BIU87-Bcl-2 cells which over-expressing Bcl-2 displayed a significant increase in the activity of NF-κB compared with uninfected control cells or those infected with a control plasmid.

Since NF-κB activity is largely governed by IκBα, which sequesters NF-κB in the cytoplasm, we determined whether the observed increase in nuclear NF-κB binding activity is related to decrease IκBα protein content. Protein extracts of BIU87 cell were subjected to western blot analysis and probed with a rabbit antibody directed toward IκBα. IκBα levels were profoundly suppressed in BIU87/Bcl-2 cells compared with BIU87 or BIU87/neo cells. These observations suggested that the increased NF-κB expression in BIU87/Bcl-2 cell may be a consequence of the enhanced degradation of IκBα, and Bcl-2 activated the transcription factor NF-κB through the degradation of the cytoplasmic inhibitor IκBα.

The mechanism by which Bcl-2 led to the activation of NF-κB was unknown but might be related to inactivation of cytoplasmic inhibitor protein IκBα. It is tempting to speculate that Bcl-2 may modulate IκBα by interacting with a variety of cellular proteins including calcineurin, Raf-1, Bag-1, Bax, and others that either directly or indirectly activate NF-κB (Bose et al., 2009; Nakamura-Yanagidaira et al., 2011). Alternatively, Bcl-2 may influence NF-κB by altering the activity of IkB kinases (Anchoori et al., 2010).

Although a direct requirement for activation of NF-κB for suppression of apoptosis by Bcl-2 was not proven, our data nevertheless suggest a tentative link between Bcl-2 and the NF-κB signaling pathway for rescue from apoptosis.
References


