RESEARCH ARTICLE

Knockdown of Bcl-3 Inhibits Cell Growth and Induces DNA Damage in HTLV-1-infected Cells)

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Abstract

Oncoprotein Bcl-3 is perceived as an unusual member of IκB family since it can both stimulate and suppress NF-κB activation. Aberrant Bcl-3 results in increased cell proliferation and survival, suggesting a contribution to malignant potential and elevated levels of Bcl-3 have been observed in many HTLV-1-infected T cell lines and ATL cells. To investigate the specific roles of Bcl-3 in HTLV-1-infected cells, we knocked down Bcl-3 expression using shRNA and then examined the consequences with regard to DNA damage and cell proliferation, as well as NF-κB activation. The HTLV-1 encoded protein Tax promotes Bcl-3 expression and nuclear translocation. In HTLV-1-infected cells, Bcl-3 knockdown obviously induced DNA damage. Cell growth and NF-κB activation were reduced in HTLV-1-infected or Tax positive cells when Bcl-3 expression was decreased. Together, our results revealed positive roles of Bcl-3 in DNA stabilization, growth and NF-κB activation in HTLV-1-infected cells.

Keywords: Bcl-3 - HTLV-1 - DNA damage - NF-κB activation

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Introduction

B-cell leukemia/lymphoma 3 (Bcl-3) is a proto-oncogene and was initially identified from the patients with chronic lymphocytic leukemia (B-CLL) (McKeithan et al., 1987; Ohno et al., 1990). Elevated Bcl-3 was found in a number of solid tumors, as well as in leukemia and lymphoma. Increased Bcl-3 expression in cancer cells leads to increased cell survival, proliferation, and malignancy (Courtois et al., 2006). Overexpression of Bcl-3 is sufficient to transform NIH3T3 cells (Viatour et al., 2004) and T cells (Valenzuela et al., 2005), suggesting an oncogenic potential. Bcl-3 was identified as an inhibitor of NF-κB (IκB) protein because of the 7 internal conserved ankyrin repeat domains within this protein. Unlike other IκB family members, Bcl-3 is predominantly localized in the nuclei and involved in the regulation of nuclear NF-κB activities. Bcl-3 is preferentially associated with NF-κB p50 and p52 homodimers (Bours et al., 1993) and can both activate (Na et al., 1998) or repress (Wessells et al., 2004) transcriptions of NF-κB-dependent genes through its interaction with coactivatiors or corepressers. Hepatitis B virus X protein (HBX) mediated p52/Bcl-3 activation induces the upregulation of cyclin D1, which leads to the increased of cell proliferation (Park et al., 2006). Bcl-3 inhibits the p53 response to DNA damage and p53-induced apoptosis through the upregulation of Hdm2 (Kashatus et al., 2006). Overexpressed Bcl-3 in activated T cells with result in higher cell survival, whereas T cells that lack Bcl-3 expression die fast (Bauer et al., 2006; Rangelova et al., 2008).

Human T cell leukemia virus type 1 (HTLV-1) is an aggressive human retrovirus and can cause adult T cell leukemia (ATL) which is an aggressive and fatal malignancy of mature CD4+ T lymphocytes. Tax, encoded by HTLV-1, plays a primary role in the pathophysiology of ATL through altering the expression of numerous genes associated with cell proliferation, immune and inflammatory response, apoptosis, and other biological processes (Harhaj et al., 2005; Shvarzbeyn et al., 2011; Yasunaga et al., 2011). Previous studies have correlated HTLV-1 infection and elevated Bcl-3 expression (Hishiki et al., 2007; Saito et al., 2010). High level of Bcl-3 was detected in many HTLV-1-infected cell lines and ATL cells, and could be transcriptionally activated by Tax protein and HTLV-1 infection (Kim et al., 2008). However, the roles of elevated Bcl-3 in HTLV-1-infected and Tax positive cells have not been fully discovered. In this study, we investigated the effects of Bcl-3 knockdown on DNA damage, cell growth, and Tax-induced NF-κB activation in HTLV-1-infected cells. We confirmed that Bcl-3 was overexpressed in HTLV-1-infected cells and found that Bcl-3 play positive roles in the regulation of DNA stabilization, cell growth and NF-κB activation in HTLV-1-infected cells. Notably, these data reveal multiple positive roles of Bcl-3 on the transformation of infected T cells and the development of ATL.

Materials and Methods

Antibodies and reagents

Anti-Bcl-3 rabbit polyclonal antibody (pAb), anti-p65 rabbit pAb and anti-Tax mouse monoclonal antibody (mAb) were bought from Santa Cruz Biotechnology

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(USA). The anti-β-actin mouse mAb, as well as the following immunoglobulin G (IgG) reagents: horseradish peroxidase-linked goat anti-rabbit, goat anti-mouse, and the FITC-conjugated goat anti-rabbit, were purchased from Zhongshan Goldenbridge Biotechnology (China). 3- (4, 5-Dimethylthiazol-2-yl) -2, 5- diphenyltetrazolium bromide (MTT) was obtained from Beyotime (China). Bcl-3 shRNA plasmid was purchased from Santa Cruz Biotechnology (USA). pNF-κB-luc, pCMV-Bam-Tax and pCMV-Bam plasmids were kindly provided by Haojiang Luan and Shoji Yamaoka, respectively.

Cell culture and transfection

HTLV-1-uninfected T cell lines Jurkat, IM9, THP-1, and HTLV-1-infected T cell lines MT2, MT4 were used in the present study. These cells were maintained in RPMI-1640 medium supplemented with 10-15% heat-inactivated fetal bovine serum, glutamine, and antibiotics at 37 °C in 5% CO_2. The transfections were performed using the Tfx-50 transfection reagent (Promega, USA) following the manufacturer's instruction.

RT-PCR assay

Total RNA was extracted with TRIZOL regent and cDNA was generated with Omniscript RT Kit (Qiagen), followed by polymerase chain reaction (PCR) assays. cDNA of cells were examined for their expression of Bcl-3 and GAPDH by PCR. Prime pairs were as follows: human GAPDH, forward 5'- TTAGCACCCCTGGCCAAGG, reverse 5'-CTTACTCCTTGGAGGCCATG; human Bcl-3, forward 5'-AACAACCTACGGCAGACACC, reverse 5'-GCGGTGAGCCC GTCATAAT.

Western blot analysis

Whole cell lysates were extracted from cells suspended in radio immune precipitation buffer (Beyotime, China) supplemented with 1 mM PMSF (Beyotime, China). The lysates were resolved by electrophoresis on polyacrylamide gels containing 0.1% SDS (SDS-PAGE) and then transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in Tris Buffer Saline Tween 20 (TBST) buffer. The blots were incubated with the appropriate primary antibody diluted by TBST and then exposed to the appropriate second antibody conjugated with horseradish peroxidase after being washed with TBST. The bands on the membrane were visualized and captured using the ECL reagent (Beyotime, China) and X-ray films (Kodak, USA).

Immunofluorescence

The cells were harvested and washed with PBS twice, and then fixed with chilled 95% ethanol. The fixed cells were washed with TBS and transferred to a slide. The cells were blocked with TBS containing 5% BSA and washed twice with TBS containing 0.1% Triton X-100. The cells were incubated overnight with TBS containing anti-Bcl-3 or anti-p65 rabbit pAb at 4 °C. The cells were washed twice with TBS containing 0.1% Triton X-100 and incubated with TBS containing FITC-conjugated goat anti-rabbit IgG. The nuclei were stained with 10μg/ml DAPI. The stained cells were washed twice with PBS and observed using the Olympus FluoView™ FV1000 microscope. The images were processed with FV10-ASW1.6 Olympus software.

Comet assay (single cell gel electrophoresis assay)

MT2 cells were transfected with Bcl-3 shRNA or control shRNA plasmids for 48 h and then harvested. Cell pellet was washed once and resuspended in PBS. Glass slides were prepared with three layers: (1) 0.5% agarose; (2) a cell suspension and 0.7% low melting point agarose mixture; (3) 0.7% low melting point agarose. These three layers were solidified in sequence at 4 °C, and subjected afterwards to a lysis step (1-hour 4 °C incubation in 1% N-laurylsarcosin, 2.5 M NaCl, 100mM Na2EDTA, 1% Triton X-100 and 10% dimethylsulfoxide). The slides were placed for 50 min in the dark, in an ice-cold electrophoresis chamber containing alkaline electrophoresis buffer to allow DNA denatured. The electrophoresis was carried out for 15 min at 25 V and 300 mA. After electrophoresis, the slide was removed from the container and allow to air dry, and then incubated in the presence of 5 0 μL/well of Propidium Iodide (PI). The slide was observed and the images were captured using fluorescence microscopy.

MTT assay

MT2 cells were transfected with Bcl-3 shRNA or control shRNA plasmids and then incubated for 48 h. 20 μl MTT (5 mg/mL) was added and the cells were incubated for an additional 4 h. The purple-blue MTT formazan precipitate was dissolved in DMSO. The activity of the mitochondria was evaluated by measuring the optical density at 570 nm. All MTT assays were performed in triplicate and repeated in three independent experiments.

Luciferase reporter assay

The cells were transfected using pNF-κB-luc plasmids and Bcl-3 shRNA plasmids. The enzymatic activities were assayed in cell extracts at 48h post-transfection using the Luciferase Assay System (Promega, USA) and 20/20 n Luminometer (Turner BioSystems, USA) according to the manufacturer's instructions. The luciferase activity was expressed as the fold of the relevant control of each experiment. All reporter assays were performed in triplicate and repeated in three independent experiments.

Data analysis

Statistical significance for the luciferase reporter assays was determined using the Student's t-test or one way ANOVA, and a p value of <0.05 was regarded as statistically significant. The standard errors were demonstrated by the bar in the figures.

Results

Overexpression of Bcl-3 in HLTV-infected cells

Bcl-3 plays an important in cell proliferation, cell survival, and exhibits malignant potential. We performed RT-PCR and Western blot to examine the mRNA and protein levels of Bcl-3 in three HTLV-1-uninfected cell lines and two HTLV-1-infected cell lines. Elevated mRNA and protein level of Bcl-3 were detected in HTLV-

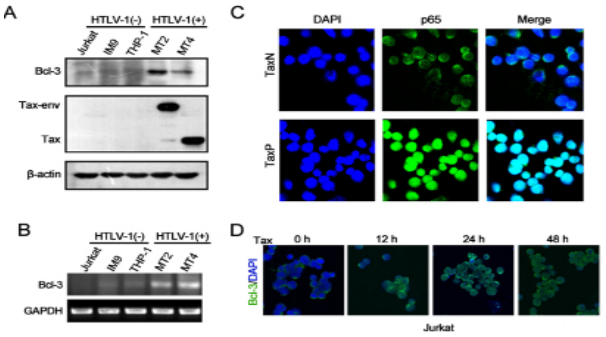

Figure 1. Bcl-3 Levels in HTLV-1 or Tax Positive Cells. (A) Whole cell lysates were extracted from 5 cell lines and assessed by Western blot for Bcl-3. Analysis of β-actin protein was included as a loading control. (B) Bcl-3 mRNA expression in five cell lines was assessed by RT-PCR. (C) The subcellular localizations of p65 (Green) in TaxN and TaxP cells were visualized with anti-p65 and FITC-conjugated anti-rabbit IgG. The nuclei was stained with DAPI (Blue). (D) Jurkat cells were transfected with Tax-expressing plasmids for indicated times, and the subcellular localizations of Bcl-3 (Green) were visualized with anti-Bcl-3 and FITC-conjugated anti-rabbit IgG. The nuclei was stained with DAPI (Blue)

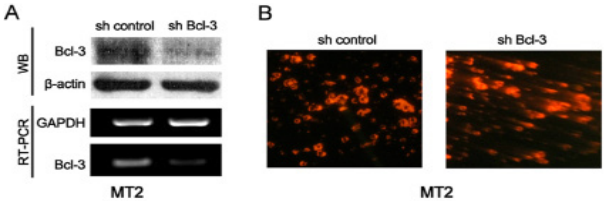

Figure 2. Bcl-3 Knockdown Promotes DNA Damage. (A) A total of 0.5 μg of Bcl-3 shRNA or control shRNA plasmids was transfected into MT2 cells. Bcl-3 protein was measured by Western blot analysis. Analysis of β-actin protein was included as a loading control. (B) After 48 h of 0.5 μg Bcl-3 shRNA or control shRNA transfection in MT2 cells, DNA damage was measured using the comet assay

1-infected cell lines MT2 and MT4 (Figure 1A and B). Whereas, Bcl-3 protein was barely detected in HTLV-1-negetive cell lines Jurkat, IM9, and THP-1. Tax-induced p65 nuclear translocation acts as a major cause in the increased NF-κB activity (O'Mahony, 2004; Sun et al., 1994). In the present study, p65 nuclear translocation was observed in TaxP cell line, which continuously expresses Tax (Figure 1C). Increased Bcl-3 expression and nuclear translocation were also observed in Tax-expressing Jurkat cells (Figure 1D). These results confirmed the high level of Bcl-3 in HTLV-1-infected cells and that Tax could induce p65 and Bcl-3 nuclear translocation.

Knockdown of Bcl-3 leads to DNA damage

Several studies have suggested a link between Bcl-3 and DNA damage response (Boulton et al., 2002; Watanabe et al., 2003). It has been shown that Bcl-3 is transiently upregulated by DNA damage and that ectopic expression of Bcl-3 suppresses DNA damage-induced apoptosis (Kashatus et al., 2006). Considering that Bcl-3 is highly expressed in HTLV-1-infected cells and the close relationship between Bcl-3 and DNA damage, we next investigated the outcome of Bcl-3 knockdown on the DNA damage in the HTLV-1-infected cells. The competent knockdown of the endogenous Bcl-3 by

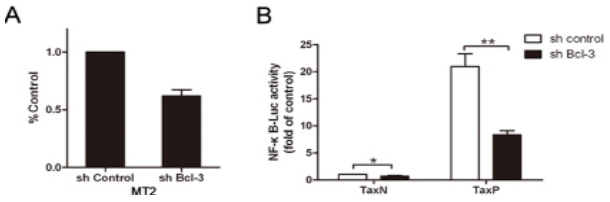

Figure 3. Bcl-3 Knockdown Inhibits the cell Proliferation and NF-κB Activation in Tax Positive Cells. (A) 0.5μg Bcl-3 shRNA plasmids were transfected into MT2 cells and the cell growth was measured using the MTT assay after transfection. (B) TaxN and TaxP cells were transfected using 0.1μg pNF-κB-luc along with or without 0.4μg Bcl-3 shRNA plasmids. The cell lysates were extracted for the luciferase activity assay after 48 h of transfection. The results from three independent experiments are shown as means ± SD. $p < 0.05$ and $p < 0.01$ were indicated by single and double asterisks, respectively

shRNA plasmids was checked by Western blot and RT-PCR analysis. Bcl-3 shRNA remarkably reduced Bcl-3 mRNA and protein expression in MT2 cells (Figure 2A). Bcl-3 could be activated by UV-induced DNA damage (Kashatus et al., 2006) and depletion of Bcl-3 leads to centrosome amplification which is associated with a DNA damage response (Zamora et al., 2010). We performed comet assay to evaluate the effect of Bcl-3 knockdown on DNA damage. DNA damage was clearly observed in the HTLV-1-infected cells transfected with Bcl-3 shRNA (Figure 2B), as the DNA tails were evidently observed in the cells transfected with Bcl-3 shRNA plasmids (Figure 2B).

Knockdown of Bcl-3 inhibits the cell growth and NF-κB activation

Bcl-3 upregulates cyclin D1 expression through NF-κB site within cyclin D1 promoter, leading to the high level of cell proliferation. In this study, we measured the cell growth of HTLV-1-infected cells transfected with Bcl-3 shRNA. As expected, cell growth was reduced upon the downregulation of Bcl-3 by shRNA in MT2 cells (Figure 3A). It has been proved that Bcl-3 acts as a pleiotropic regulator in NF-κB signaling pathway. We utilized the luciferase reporter assay to examine the effect of Bcl-3 knockdown on NF-κB activation in the presence or absence of Tax (Figure 3B). Downregulation of Bcl-3 inhibits the NF-κB activation in both TaxN and TaxP cells, but the inhibition of NF-κB activity in TaxP cells was more significant compared with that in TaxN cells. These data indicated the positive roles of Bcl-3 in cell growth and NF-κB activation in HTLV-1 positive cells.

Discussion

Elevated Bcl-3 levels in many HTLV-1-infected and ATL cells were explored in several studies. Tax protein is directly responsible for the transcription of Bcl-3 (Kim et al., 2008) and a post-transcriptional mechanisms associated with PI3K-Akt pathway is also involved in the accumulation of Bcl-3 protein (Saito et al., 2010). In this study, we first confirmed the elevated mRNA and protein levels of Bcl-3 in HTLV-1 positive cell lines. Bcl-3 overexpression and nuclear translocation were also found

in the presence of Tax protein. Expression of Tax is also responsible for the persistent NF-κB activation and NF-κB p65 nuclear translocation. Kim et al. have indicated that Tax induces Bcl-3 expression primarily through activation of NF-κB pathway (Kim et al., 2008). In the present study, p65 nuclear translocation was observed in TaxP cells which continuously express Tax protein.

Previous study has shown that recruitment of certain DNA damage response proteins also occurs during infection (Everett, 2011), suggesting the infection could lead to the DNA damage. p53 functions as a crucial player in the maintenance of genome integrity through the regulation of cell response to stress such as DNA damage and oncogene activation. Bcl-3 expression could inhibit p53-induced apoptosis and we found the DNA damage was induced by Bcl-3 knockdown in this study.

Upregulation and nuclear localization of Bcl-3 can increase the p52/Bcl-3 complex in the NF-κB site of cyclin D1 promoter, leading to the upregulation of cyclin D1 which involved in the regulation of cell growth (Park et al., 2006). Overexpression of Bcl-3 also inhibits p53-induced apoptosis through a mechanism that is at least partly dependent on the upregulation of Hdm2 (Kashatus et al., 2006). Considering the multiple functions of Bcl-3, the abnormal expression of this protein in HTLV-1-infected cells and ATL cells contribute to the cell proliferation and survival. Our finding reveals that Bcl-3 plays a positive role in cell growth of HTLV-1-infected cells and contributes to the NF-κB activation induced by Tax. Thus, Tax-induced Bcl-3 may promote cell growth through the regulation of cyclin D1 and increase NF-κB activity through the interaction with p50 or p52 homodimers.

In conclusion, the present study shows that the knockdown of Bcl-3 in HTLV-1 or Tax positive cells induces DNA damage, inhibition of cell growth and NF-κB activation. Bcl-3 may contribute to the ATL development through the positive regulation of cell growth, DNA stabilization and NF-κB activation.

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References

Bauer A, Villunger A, Labi V, et al (2006). The NF-kappaB regulator Bcl-3 and the BH3-only proteins Bim and Puma control the death of activated T cells. *Proc Natl Acad Sci U S A* , **103**, 10979-84.

Boulton SJ, Gartner A, Reboul J, et al (2002). Combined functional genomic maps of the C. elegans DNA damage response. *Science*, **295**, 127-31.

Bours V, Franzoso G, Azarenko V, et al (1993). The oncoprotein Bcl-3 directly transactivates through kappa B motifs via association with DNA-binding p50B homodimers. *Cell*, **72**, 729-39.

Courtois G, Gilmore TD (2006). Mutations in the NF-kappaB signaling pathway: implications for human disease. *Oncogene*, **25**, 6831-43.

Everett RD (2011). The use of fluorescence microscopy to study the association between herpesviruses and intrinsic resistance factors. *Viruses*, **3**, 2412-24.

Harhaj E, Harhaj N (2005). Mechanisms of Persistent NF-κB Activation by HTLV-I Tax. *IUBMB Life*, **57**, 83-91.

Hishiki T, Ohshima T, Ego T, et al (2007). BCL3 acts as a negative regulator of transcription from the human T-cell leukemia virus type 1 long terminal repeat through interactions with TORC3. *J Biol Chem*, **282**, 28335-43.

Kashatus D, Cogswell P, Baldwin AS (2006). Expression of the Bcl-3 proto-oncogene suppresses p53 activation. *Genes Dev*, **20**, 225-35.

Kim Y.M., Sharma N, Nyborg JK (2008). The proto-oncogene Bcl3, induced by Tax, represses Tax-mediated transcription via p300 displacement from the human T-cell leukemia virus type 1 promoter. *J Virol*, **82**, 11939-47.

McKeithan TW, Rowley JD, Shows TB, et al (1987). Cloning of the chromosome translocation breakpoint junction of the t(14;19) in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A*, **84**, 9257-60.

Na SY, Choi HS, Kim JW, et al (1998). Bcl3, an IkappaB protein, as a novel transcription coactivator of the retinoid X receptor. *J Biol Chem*, **273**, 30933-8.

O'Mahony AM (2004). Human T-cell Lymphotropic Virus Type 1 Tax Induction of Biologically Active NF- B Requires I B Kinase-1-mediated Phosphorylation of RelA/p65. *J Biol Chem*, **279**, 18137-45.

Ohno H, Takimoto G, McKeithan TW (1990). The candidate proto-oncogene bcl-3 is related to genes implicated in cell lineage determination and cell cycle control. *Cell*, **60**, 991-7.

Park SG, Chung C, Kang H, et al (2006). Up-regulation of cyclin D1 by HBx is mediated by NF-kappaB2/BCL3 complex through kappaB site of cyclin D1 promoter. *J Biol Chem*, **281**, 31770-777.

Rangelova S, Kirschnek S, Strasser A, et al (2008). FADD and the NF-kappaB family member Bcl-3 regulate complementary pathways to control T-cell survival and proliferation. *Immunology*, **125**, 549-57.

Saito K, Saito M, Taniura N, et al (2010). Activation of the PI3K-Akt pathway by human T cell leukemia virus type 1 (HTLV-1) oncoprotein Tax increases Bcl3 expression, which is associated with enhanced growth of HTLV-1-infected T cells. *Virology*, **403**, 173-80.

Shvarzbeyn J, Huleihel M (2011). Effect of propolis and caffeic acid phenethyl ester (CAPE) on NFκB activation by HTLV-1 Tax. *Antiviral Res*, **90**, 108-15.

Sun SC, Elwood J, Beraud C, et al (1994). Human T-cell leukemia virus type I Tax activation of NF-kappa B/Rel involves phosphorylation and degradation of I kappa B alpha and RelA (p65)-mediated induction of the c-rel gene. *Mol Cell Biol*, **14**, 7377-84.

Valenzuela JO, Hammerbeck CD, Mescher MF (2005). Cutting edge: Bcl-3 up-regulation by signal 3 cytokine (IL-12) prolongs survival of antigen-activated CD8 T cells. *J Immunol*, **174**, 600-4.

Viatour P, Dejardin E, Warnier M, et al (2004). GSK3-mediated BCL-3 phosphorylation modulates its degradation and its oncogenicity. *Mol Cell*, **16**, 35-45.

Watanabe N, Wachi S, Fujita T (2003). Identification and characterization of BCL-3-binding protein: implications for transcription and DNA repair or recombination. *J Biol Chem*, **278**, 26102-10.

Wessells J, Baer M, Young HA, et al (2004). BCL-3 and NF-kappaB p50 attenuate lipopolysaccharide-induced inflammatory responses in macrophages. *J Biol Chem*, **279**, 49995-50003.

Yasunaga J, Lin FC, Lu X, et al (2011). Ubiquitin-Specific Peptidase 20 Targets TRAF6 and Human T Cell Leukemia Virus Type 1 Tax To Negatively Regulate NF- B Signaling. *J Virol*, **85**, 6212-9.

Zamora R, Espinosa M, Ceballos-Cancino G, et al (2010). Depletion of the oncoprotein Bcl-3 induces centrosome amplification and aneuploidy in cancer cells. *Mol Cancer*, **9**, 223.