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Original Contribution

Nrf2-induced antiapoptotic Bcl-xL protein enhances cell survival and drug resistance

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ABSTRACT

Nuclear transcription factor Nrf2 binds with the antioxidant-response element (ARE) in the promoter regions of cytoprotective genes, leading to their increased expression and cellular protection. In this study, we investigated the role of Nrf2 in the regulation of antiapoptotic Bcl-xL protein and its effect on cellular apoptosis. Treatment of mouse Hepa-1 cells with the antioxidant tert-butylhydroquinone led to the induction of Bcl-xL gene expression. Promoter mutagenesis, transfection, and chromatin immunoprecipitation assays identified an ARE between nucleotides −608 and −600 in the forward strand of the proximal Bcl-xL promoter that bound to Nrf2 and led to increased Bcl-xL gene expression. In addition, short interfering RNA (siRNA) inhibition and overexpression of Nrf2 led to a respective decrease and increase in Bcl-xL gene expression. These results implicated Nrf2 in the regulation of expression and induction of Bcl-xL protein. Nrf2-mediated expression of Bcl-xL protein downregulated Bax and decreased caspase 3/7 activity. SiRNA inhibition of both Nrf2 and Bcl-xL increased the susceptibility of cancer cells to etoposide-mediated cell death and reduced cell survival. Moreover, dysfunctional/mutant INrf2 (inhibitor of Nrf2) in human lung cancer cells failed to degrade Nrf2, resulting in increased Bcl-xL levels and increased cell survival. These data provide the first evidence of Nrf2 in the control of Bcl-xL expression and apoptotic cell death with implications for antioxidant protection, survival of cancer cells, and drug resistance.

The Nrf2 (NF-E2 related factor 2):INrf2 (inhibitor of Nrf2) complex serves as a sensor of chemical- and radiation-induced oxidative and electrophilic stress [1,2]. Nrf2 resides predominantly in the cytoplasm, where it interacts with the actin-associated cytosolic protein INrf2, or Keap1 (Kelch-like ECH-associated protein 1). INrf2 functions as a substrate adaptor protein for a Cul3–Rbx1-dependent E3 ubiquitin ligase complex to ubiquitinate and degrade Nrf2, thus maintaining a steady-state level of Nrf2 [1]. The mechanisms by which Nrf2 is released from INrf2 under stress have been actively investigated. In one mechanism cysteine thiol groups of INrf2 were shown to function as sensors for oxidative stress that are modified by chemical inducers, causing formation of disulfide bonds between cysteines of two INrf2 peptides. This results in a conformational change that renders INrf2 unable to bind to Nrf2 [2,3]. On the other hand, we and others have shown that antioxidant-induced phosphorylation of Nrf2 serine40 by protein kinase Cδ leads to dissociation of Nrf2 from INrf2 [4,5]. Nrf2 stabilizes and translocates to the nucleus, leading to induction of downstream cytoprotective proteins [4,5]. Recent studies have shown that the two mechanisms act in concert to activate Nrf2 and downstream genes in response to chemical and radiation stress [6].

The antioxidant-response element (ARE) is a cis-acting regulatory element in the promoter regions of several genes encoding phase II detoxification enzymes and antioxidant proteins, such as NAD(P)H:quinine oxidoreductase-1 (NQO1), glutathione S-transferase, γ-glutamylcysteine synthetase, heme oxygenase-1, thioredoxin reductase-1, and thioredoxin [1]. Transcriptional activation through the ARE is mainly regulated by Nrf2. Recently, Nrf2 and its downstream proteins have been shown to be critical regulators in protection of cells from oxidative stress- and chemical-induced damage to liver and lung tissues. It has been demonstrated that Nrf2 knockout mice are more sensitive to hyperoxic injury of lung [7]. The primary astrocyte of $Nrf2^{-/-}$ mice is also more susceptible to oxidative stress and inflammation than that of $Nrf2^{+/+}$ mice [8,9]. Leung et al. [9] showed that deficiency of Nrf2 results in early embryonic lethality with severe oxidative stress. These observations, collectively, imply that Nrf2 is a master regulator of ARE-driven transcriptional activation for antioxidant genes in maintaining the homeostasis of the redox status within cells. In contrast, evidence also suggests that persistent accumulation of Nrf2 in the nucleus is harmful. INrf2-null mice demonstrated persistent accumulation of Nrf2 in the nucleus that led to

Abbreviations: Nrf2, NF-E2 related factor 2; INrf2 (Keap1), inhibitor of Nrf2; ARE, antioxidant-response element; tBHQ, tert-butylhydroquinone

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postnatal death from malnutrition resulting from hyperkeratosis in the esophagus and forestomach [10]. Reversal of the phenotype of INrf2 deficiency by breeding to Nrf2-null mice suggested that tightly regulated negative feedback might be essential for cell survival [11]. The systematic analysis of the INrf2 genomic locus in human lung cancer patients and cell lines showed that deletion, insertion, and missense mutations in functionally important domains of INrf2 result in reduction of INrf2 affinity for Nrf2 and elevated expression of cytoprotective genes, which resulted in drug resistance and cell survival in lung cancer cells [12,13]. Unrestrained activation of Nrf2 in cells increases the risk of adverse effects including survival of damaged cells, tumorigenesis, and drug resistance [6]. Therefore, it seems that cells contain mechanisms that autoregulate the cellular abundance of Nrf2 [14,15]. Indeed, these results suggested that Nrf2 plays an important role in cell survival/protection in normal cells and drug resistance in cancer cells.

In cancer, apoptosis is a critical process that is dysregulated, resulting in tumorigenesis [16]. Bcl-2 family proteins regulate cell death and survival [17,18]. Bcl-2 family proteins are the prototypical antiapoptotic proteins, and Bcl-xL was the first protein discovered with a similar function [19]. Since then the Bcl-2 family has expanded to include more than six antiapoptotic and many proapoptotic members [20]. Bcl-2 and Bcl-xL display 43% amino acid identity and share regions of sequence similarity, as well as a C-terminal hydrophobic region required for membrane localization [21]. Bcl-2 and Bcl-xL appear to function in the same apoptotic pathway and both confer resistance to multiple chemotherapy agents when tested in experimental systems. Overexpression of either protein is usually associated with poor prognosis in many human cancers. However, in some cancer types multiple antiapoptotic proteins are expressed [22] and have opposing effects on prognosis, indicating that there may be subtle, but clinically and biologically relevant, functional differences between family members. Experiments in mice with deletion of individual antiapoptotic genes indicate that the phenotypes are not identical [23]. However, it is generally accepted that this is due to expression in different tissues or in the same tissue but at different times, rather than being a consequence of differences in the potency or mechanism of action of the different antiapoptotic proteins. The mechanisms of action of Bcl-2 and Bcl-xL are complex, with many postulated interactions with other proteins, and the role of any single interaction in the final phenotype at the cellular level remains unknown.

Several studies have investigated the INrf2:Nrf2 regulation of antiapoptotic proteins and their role in cellular apoptosis especially during chemical and radiation stress. H2S-mediated stabilization of Nrf2 increased the levels of the antiapoptotic proteins Bcl-2 and Bcl-xL, which led to cardioprotection in mice [24]. INrf2-mediated degradation of antiapoptotic Bcl-2 and Bcl-xL proteins contributed to increased cellular apoptosis [25,26]. Nrf2-regulated Bcl-2 expression also prevented cellular apoptosis under stress conditions that led to cell survival [27]. However, the role of Nrf2 in the regulation of Bcl-xL gene expression and induction during stress remains unknown.

In this study, we investigated the role of Nrf2 in the regulation of the antiapoptotic factor Bcl-xL and its contribution to cellular apoptosis. Deletion mutagenesis of the Bcl-xL gene promoter identified an antioxidant-response element in the forward strand of the proximal Bcl-xL promoter that bound to Nrf2 and activated Bcl-xL gene expression. Nrf2-mediated upregulation of Bcl-xL downregulated the proapoptotic protein Bax, reduced caspase 3/7 activity, and protected cells from etoposide-mediated apoptosis. These results led to the conclusion that Nrf2-mediated induction of Bcl-xL plays an important role in decreasing cellular apoptosis and enhancing cell survival.

Materials and methods

Plasmid construction

The 1.565-kb mouse Bcl-xL gene promoter was isolated from mouse tail genomic DNA by PCR using the forward 5′-ATTCTTGCTAGCTAGTGTCTGGAAGCCACTGGG-3′ and reverse 5′-ACCGCCAGATCTGCCTGTGTTTAGCGATTCTCTTC-3′ set of primers and High-Fidelity Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The PCR-amplified promoter fragment was cloned into the pGL2-Basic luciferase vector (Promega, Madison, WI, USA) using NheI and BglII restriction sites. The resulting plasmid was designated as pGL2b−1.565-kb (−64 to −1565, +1 is the ATG site). Two deletion plasmids of the Bcl-xL promoter were generated using a specific set of primers: forward primers 5′-ATTATTGCTAGCTGGCTGGAGCCTGGAGCAGAGA-3′ (for −0.650 kb plasmid) and 5′-ATTATTGCTAGCTTCGCAATTCCTCTGTCGCCTTCT-3′ (for −0.588 kb plasmid) and the same reverse primer, 5′-ACCGCCAGATCTGCCTGTGTTTAGCGATTCTCTTC-3′, were used to generate Bcl-xL promoter deletion plasmids. Forward primer 5′-GATGGAGGAACCAGGTTGACTGGGGATAGGTTCCTAAG-3′ and reverse primer 5′-CAACCTGGTTCCTCCATCGACCAGATCGAGGGCGGC-3′ and the Gene Tailor site-directed mutagenesis kit (Invitrogen) were used to generate the mutant ARE-F1 plasmid. In addition, we generated pGL2p-ARE-F1 and mutant ARE-F1 plasmids. ARE-F1 oligonucleotides (plus strand 5′-ATTGCACCCGGGGCTAGCCAGGTTGTGAGGGGGCAGGTTCCT-3′ and minus strand 5′-ATTCGGCCCGGGGCTAGCAGGAACCTGCCCCCTCACAACCTG-3′) were synthesized, annealed, digested with SmaI and NheI enzymes, and cloned into the pGL2p vector to generate the pGL2p-ARE-F1 plasmid. Similarly, mutant ARE-F1 oligonucleotides (plus strand 5′-ATTGCACCCGGGGCTAGCCAGGTTGAATGGGGTTAGGTTCCT-3′ and minus strand 5′-ATTCGGCCCGGGGCTAGCAGGAACCTAACCCCATTCAACCTG-3′) were cloned into pGL2p to generate pGL2p-mutant ARE-F1. We replaced the luciferase coding sequence in wild-type pGL2b-1.565-WT and ARE-F1 mutant pGL2b-1.565-MT plasmids with Bcl-xL cDNA using BglII and ClaI sites. The Bcl-xL coding sequence was PCR amplified using forward primer 5′-ATTCGAAGATCTACCGCCATGTCTCAGAGCAACCGG-3′ and reverse prime 5′-TTACATATCGATCTACTTCCGACTGAAGAGTGAGCCCAG-3′. The sequence accuracy of all plasmids was confirmed by DNA sequencing using an ABI3700 capillary sequencer (Applied Biosystems, Foster City, CA, USA). The construction of a luciferase plasmid harboring the human NQO1 gene ARE and pCMV-Flag-Nrf2, pCMV-Flag-INrf2, and pcMV-Flag-Bcl-xL plasmids was described previously [26,28].

Cell culture and generation of stable Flp-In T-REx HEK293 cells expressing tetracycline-inducible Nrf2 and INrf2

Mouse hepatocarcinoma (Hepa-1) and human hepatoblastoma (HepG2) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Human embryonic kidney (HEK293) cells were obtained from Invitrogen. Hepa-1 and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (40 U/ml), and streptomycin (40 μg/ml). HepG2 cells were grown in α-minimum essential medium containing 10% fetal bovine serum, penicillin (40 U/ml), and streptomycin (40 μg/ml). INrf2 mutant lung cancer A549 cells were grown in F12/DMEM. We also generated wild-type INrf2-expressing stable A549 cells by transfection of pcDNA-INrf2 followed by selection of clones with neomycin (G148). For generation of stable Nrf2 and INrf2-expressing cells, Flp-In T-REx HEK293 cells were purchased from Invitrogen and cotransfected with Flag-Nrf2 or Flag-INrf2 in pcDNA/FRT/TO plasmids along with pOG44 plasmid (Invitrogen) using the Effectene method (Qiagen, Valencia, CA, USA) and the