Silencing of Ref-1 Expression by Retrovirus-Mediated shRNA Sensitizes HEK293 Cells to Hydrogen Peroxide-Induced Apoptosis

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Redox factor-1 (Ref-1) is a multifunctional protein involved in DNA base excision repair (BER) and transcription factor modification. By the use of retrovirus-delivered shRNA, we effectively suppressed endogenous Ref-1 expression in human embryonic kidney (HEK) 293 cells. Our results showed that downregulation of Ref-1 rendered cells more sensitive to H2O2-induced apoptosis. In this process, the absence of Ref-1 decreased the ratio of Bcl-2/Bax protein expression, which resulted in cytochrome c release and caspase-3 activation. These data indicate that constitutive Ref-1 is required for cellular defense and that this protective function is dependent on its role in the regulation of Bcl-2 family proteins.

Key words: redox factor-1; RNA interference; oxidative stress; Bcl-2; Bax

Ref-1 is ubiquitously expressed and highly induced in response to a variety of cellular stresses. As a multifunctional protein, it is involved in BER and gene expression regulation.1) Through redox activity Ref-1 can modify a number of transcription factors, including AP-1, p53, NF-κB, and HIF-α.2–5) Overexpression of Ref-1 has been implicated in protection against cell death resulting from oxidant, chemotherapeutic drugs, and radiation treatment.6–8) and downregulation of its level renders cells more sensitive to induced apoptosis.9,10) In addition, Ref-1 is associated with tumorigenesis, cell-cycle control, and adaptive response.1) Although the DNA repair property has been well established, the mechanisms by which Ref-1 may regulate the redox state of cells have not been definitively identified.

The Bcl-2 protein is constitutively bound to outer mitochondria membranes, and its overexpression inhibits the cell death promoted by a wide variety of stimuli. Bax is another member of the Bcl-2 family, but exerts an apoptotic effect. In response to oxidative stress, Bax is translocated from the cytosol to the mitochondria, leading to increased mitochondrial permeability, the release of cytochrome c, and activation of caspases. Active Bcl-2 can form a heterodimer with Bax, thereby neutralizing its apoptotic effects. Since the Bcl-2/Bax ratio is a key factor in the regulation of oxidant-induced apoptosis,11) we examined to determine whether Ref-1 modulates the balance of Bcl-2 and Bax gene expression.

Cytochrome c is located in the mitochondrial inner membrane. Upon apoptotic stimulation, cytochrome c is released into the cytosol, and thus can favor caspase-3 activation.12) Cytochrome c release and caspase-3 activation are key steps during mitochondria-mediated apoptosis, which are tightly regulated by Bcl-2 family proteins.13)

In the present study, we constructed a novel retrovirus-based RNA interference vector to decrease Ref-1 expression effectively and permanently. HEK293 cells were usually used as the cell model in investigating oxidative stress, and were found to express high levels of Ref-1. Our data indicated that inhibition of Ref-1 sensitizes cells to H2O2-induced death by mechanisms involving alteration of Bcl-2/Bax expression, which leads to the release of cytochrome c and the activation of caspase-3.

Materials and Methods

Construction of a retroviral vector for delivery of shRNA. The retroviral vector for delivery of shRNA is based on the Pmscv/Hyg vector (Clontech, Mountain View, CA). Human U6 promoter was cloned from genomic DNA and inserted upstream of the 3′LTR. The oligonucleotides encoding the Ref-1 shRNA were
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5’-GATCCGATCTGCTTTGGAATGTGTCACACATCCAGGACGACATCTTTTTTG-3’, and 5’-TCGACAAAAAGATCTGCTTGGGATATGTCTCTTGACTACATCCAGGACGATCG-3’. Two reverse complemented oligonucleotides were annealed and ligated downstream of the U6 promoter. Control vector with no target specificity was constructed by the same method.

**Generation of virus.** PT-67 cells were transfected into plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Virus supernatant was collected 48 h post-transfection, filtered through a 0.45-μm polysulfonic filter to remove cellular debris, and added directly to HEK293 cells with polybrene (8 μg/ml final concentration). After 12 h, the medium was replaced with fresh medium. The stably infected cells were selected with hygromycin (100 μg/ml final concentration) for 14 d.

**RT-PCR.** RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA), and reverse transcription was performed at 42°C for 30 min. The PCR primers were as follows: Ref-1 (383 bp), 5’-ccg agt ctt ggt act gg-3’, and 5’-cct tgg aac gga tct tgc tg-3’; β-actin (475 bp), 5’-ggg aac tgc ttc ggg aca tta-3’, and 5’-tcg tca tcc ttc tgc tgg ctg-3’. PCR products were separated on 1.5% agarose gel and visualized by EB staining.

**Cell culture and reagent treatment.** The cells were maintained in DMEM (Gibco, California) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 10% (v/v) fetal bovine serum at 37°C with 5% CO₂. To induce moderate oxidative stress, the cells were treated with 200 μM H₂O₂ (Sigma, USA) for 30 min. After 30 min, the medium was replaced with fresh medium.

**Evaluation of apoptotic cells.** 12 h after H₂O₂ treatment, cells were harvested, washed with PBS, fixed in 70% ethanol, and stained with propidium iodide. They were examined by flow cytometry. Sub-G₁ peaks on DNA histograms from hypodiploid DNA represented apoptotic cells.

**Preparation of mitochondrial and cytosolic extracts.** Cells were harvested, washed once with ice-cold phosphate buffered saline, and gently lysed for 2 min in 80 μl ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 20 mM Tris–HCl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM MgCl₂, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). The lysates were centrifuged at 12,000 g at 4°C for 10 min to obtain supernatants and pellets. The supernatants were used in the identification of cytosolic cytochrome c by immunoblot.

**Immunoblot assays.** The cells were pelleted and then washed twice with PBS prior to lysis in sample buffer (5% glycerol, 1% SDS, 100 mM dithiothreitol, 0.2% bromphenol blue, 60 mM Tris, pH 6.8), followed by sonication and boiling. Protein concentrations were determined by Bradford assay. Protein samples were separated with 12% SDS-polyacrylamide gel and transferred onto PVDF membrane. Anti-Ref-1, anti-Bcl-2, anti-Bax, anti-Caspase-3, anti-cytochrome c, and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and the immunoblot results were visualized by ECL assay. The relative intensity of the immunoblot bands was quantified with Gene Tool Analysis Software 3.02.00 (Syngene, UK).

**Statistical analysis.** Statistical analysis was performed using SPSS software (SPSS for windows package release 11.0, SPSS, Chicago). Data were expressed as mean ± SD. A value of p < 0.05 was judged as statistically significant.

**Results**

**Retrovirus-delivered shRNA inhibited Ref-1 expression.** We constructed a novel retrovirus-based shRNA vector targeting Ref-1. The scheme of recombinant plasmid is shown in Fig. 1A. The efficacy of shRNA-mediated inhibition of Ref-1 synthesis was analyzed by

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**Fig. 1.** Suppression of Ref-1 Expression by Retrovirus-Delivered shRNA.

A. Schematic representation of the recombinant vector. Ph′, packaging signal; neo+, neomycin gene; Hgyr, hygromycin gene. Complementary oligonucleotides were annealed and inserted between BamHI and Sall in pmscv-shRNA. B. Total RNA from shRNA infected and control cells were prepared for RT-PCR to detect expression of Ref-1 mRNA. The levels of Ref-1 mRNA were normalized to that of β-actin, which was used as a loading control. C. shRNA infected and control cell extracts were harvested for Western blot analysis to detect expression of the Ref-1 protein. The average signal intensity of the Ref-1 protein was normalized to that of β-actin.
RT-PCR and Western blotting. As shown in Fig. 1B, when HEK293 cells were infected with Ref-1 shRNA, Ref-1 mRNA and proteins were downregulated by approximately 80%, but negative control shRNA did not affect the Ref-1 mRNA or protein. \(\beta\)-actin mRNA and protein levels were not affected by these shRNAs.

**Inhibition of Ref-1 sensitized cells to \(\text{H}_2\text{O}_2\)-mediated death**

Prior to \(\text{H}_2\text{O}_2\) treatment, we observed that silencing of Ref-1 did not disrupt HEK293 cell viability under normal growth conditions (data not shown). To investigate the effects of Ref-1 downregulation in \(\text{H}_2\text{O}_2\)-induced cell death, cells were exposed to a sub-lethal concentration of \(\text{H}_2\text{O}_2\) (200 \(\mu\)M) for 30 min to induce moderate oxidative stress. After 12 h stimulation, the HEK293/Ref-1-shRNA cells showed shrinkage and dissociation from the surrounding cells. In contrast, these morphological changes were rarely observed in the control cells (data not shown). Flow cytometry analysis indicated that silencing of Ref-1 increased the vulnerability of HEK293 cells to \(\text{H}_2\text{O}_2\)-induced apoptosis (Fig. 2).

**Inhibition of Ref-1 blocked Bcl-2 upregulation but promoted Bax expression in response to \(\text{H}_2\text{O}_2\)**

Bcl-2 family proteins have a central role in controlling the apoptotic pathway. In the present study, we determined the protein levels of Bcl-2 and Bax. Under culture conditions, downregulation of Ref-1 did not affect basic Bcl-2 and Bax expression. Transient \(\text{H}_2\text{O}_2\) treatment, as expected, increased the levels Bcl-2 and Bax in the parent HEK293 cells and reached a maximum at 3–4 h. In HEK293/Ref-1-shRNA cells, \(\text{H}_2\text{O}_2\) induction of Bcl-2 was suppressed, while Bax significantly increased as compared to negative control cells (Fig. 3A). The Bcl-2/Bax ratio shifted from a value of 1.8 in the control cells to about 0.8 in the HEK293/Ref-1-shRNA cells (Fig. 3B).

**Fig. 2.** Ref-1 Downregulation Sensitizes HEK293 Cells to \(\text{H}_2\text{O}_2\)-Induced Apoptosis.

Cells were treated with \(\text{H}_2\text{O}_2\) (200 \(\mu\)M) for 30 min, recovered, and analyzed for cell cycle distribution by PI staining and flow cytometric analysis. The sub-G1 peak corresponds to apoptotic cells and is indicated as apoptosis. a, parent 293 cells without \(\text{H}_2\text{O}_2\) treatment; b, parent 293 cells with \(\text{H}_2\text{O}_2\) treatment; c, HEK293/control-shRNA with \(\text{H}_2\text{O}_2\) treatment; d, HEK293/Ref-1-shRNA with \(\text{H}_2\text{O}_2\) treatment. Results represent the mean ± S.D. of triplicate experiments.

**Fig. 3.** Effects of Ref-1 on \(\text{H}_2\text{O}_2\)-Induced Bcl-2 and Bax Expression.

A. Western blot determination of Bcl-2 and Bax proteins. 4 h after \(\text{H}_2\text{O}_2\) treatment, cells were harvested for immunoblot analysis. Inhibition of Ref-1 attenuated Bcl-2 upregulation and promoted Bax increase. B, Bcl-2 and Bax band densities were measured, and Bcl-2/Bax ratios were graphed for the various conditions. Data represent the mean ± S.D. of triplicate experiments.
Inhibition of Ref-1 Promoted Cytochrome c Release and Caspase-3 Activation

The release of cytochrome c from the mitochondria to the cytosol was checked by western blot. The results showed that 12 h after H$_2$O$_2$ treatment, a clear release of cytochrome c to cytosol occurred in HEK293/Ref-1-shRNA cells as compared with the negative control cells. In addition, western blot analysis showed notable expression of active caspase-3 by the appearance of 17 kDa in HEK293/Ref-1-shRNA cells (Fig. 4). These results indicate that silencing of Ref-1 sensitization of HEK293 cells to H$_2$O$_2$-induced apoptosis is associated with mitochondria-mediated pathway.

Discussion

Oxidative stress has been identified as an important process in a variety of diseases. In response to oxidative injury, cells have adopted a number of defensive systems to maintain cellular stability, including constitutive and inducible antioxidants, enzymatic scavengers of ROS, and DNA repair enzymes. Based on DNA repair and redox modifying activities, Ref-1 has been found to be one of the key molecules in cell defense. By the use of retrovirus-delivered shRNA, we suppressed endogenous Ref-1 expression in HEK293 cells effectively and stably. Our results indicate that silencing of Ref-1 represses the redox activation of transcription factors, such as NF-$\kappa$B, p53, and AP-1, mediate Bcl-2 and Bax gene expression. Coincidentally, these transcription factors are downstream targets modified by Ref-1 through reduction of a conserved cysteine residue, which is necessary for their DNA binding activity. We speculate that silencing of Ref-1 represses the redox activation of transcription factors, and thus disturbs cellular anti-oxidant gene expression, such as Bcl-2. On the other hand, inhibition of the NF-$\kappa$B transcription factor can increase Bax expression. These fundamental mechanisms should be studied in future experiments.

Cytochrome c is located in the mitochondrial intermembrane spaces. Apoptotic stimuli can promote cytochrome c to release to the cytosol, and thus binds to Apaf-1, which is an upstream mediator of caspase-3 activation. Activation of caspase-3 is essential for apoptosis. The release of cytochrome c and caspase-3 activation are rigorously regulated by the ratio of Bcl-2/Bax. We conclude that silencing of Ref-1 stimulates cytochrome c release and caspase-3 activation in response to sublethal concentrations of H$_2$O$_2$, which results from the decreased Bcl-2/Bax ratio.
Taken together, our results suggest that constitutive expression of Ref-1 is required to trigger the defense reaction to antagonizing oxidative stress by mechanisms involving the modulation Bcl-2 family proteins. The complete molecular mechanism that coordinates these events remains to be determined.

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References