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Peroxiredoxin I and II Inhibit H_2O_2-Induced Cell Death in MCF-7 Cell Lines

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Abstract Apoptosis is known to be induced by direct oxidative damage due to oxygen-free radicals or hydrogen peroxide or by their generation in cells by the actions of injurious agents. Together with glutathione peroxidase and catalase, peroxiredoxin (Prx) enzymes play an important role in eliminating peroxides generated during metabolism. We investigated the role of Prx enzymes during cellular response to oxidative stress. Using Prx isoforms-specific antibodies, we investigated the presence of Prx isoforms by immunoblot analysis in cell lysates of the MCF-7 breast cancer cell line. Treatment of MCF-7 with hydrogen peroxide (H_2O_2) resulted in the dose-dependent expressions of Prx I and II at the protein and mRNA levels. To investigate the physiologic relevance of the Prx I and II expressions induced by H_2O_2, we compared the survivals of MCF10A normal breast cell line and MCF-7 breast cancer cell line following exposure to H_2O_2. The treatment of MCF10A with H_2O_2 resulted in rapid cell death, whereas MCF-7 was resistant to H_2O_2. In addition, we found that Prx I and II transfection enabled MCF10A cells to resist H_2O_2-induced cell death. These findings suggest that Prx I and II have important functions as inhibitors of cell death during cellular response to oxidative stress. J. Cell. Biochem. 101: 1038–1045, 2007.

Key words: Prx; H_2O_2; MCF10A; MCF-7; breast cancer cell

Oxidative stress has been implicated in the normal aging process and in numerous disorders including cancer, muscular atrophy, neurodegenerative diseases, and atherosclerosis [Cross et al., 1987; Sha et al., 1988; Halliwel et al., 1992; Sarsfian et al., 1998]. Mammalian cells have two metal catalyzed oxidative mechanisms, namely the ascorbate and the thiol oxidation systems. Both of these can be inhibited by catalase or glutathione synthetase, which protect them from reactive oxygen species (ROS) [Baker et al., 1997]. Moreover, catalase and superoxide dismutase are known to be able to protect cells from different initiators of apoptosis [Hampton and Orrenius, 1998; Ichimiya, 1997]. Hydrogen peroxide (H_2O_2), which causes direct oxidative injury to cells, is believed to be a second messenger in the cell-signaling pathway, and therefore, reductase species may play an important role in cellar phenomena such as proliferation, differentiation, and immune response.

Peroxiredoxin (Prx), reduces H_2O_2 to water and was previously called thioredoxin peroxidase. Prx is a 25-kDa protein that acts in the thiol oxidation system. It was first discovered in yeast in 1994 [Chae et al., 1994] and since then more than 100 isoforms, with some sequence homology, have been identified in species ranging from protozoa [Salinas et al., 1998; Jeong et al., 1999] to mammals, including rat, mouse, and human. Thus, Prx is believed to be of biological importance, because these molecules are highly conserved in eukaryotes and prokaryotes [Kim et al., 1996]. The gene locus for these proteins in human is regarded to be located in chromosome 13q12 [Pahl et al., 1995]. Six Prx isoforms have been reported in human, namely, NKEF-A, NKEF-B, MER5, AOE372, TAS, and ORF6 [Jin et al., 1997]. These enzymes may have functions other than reductase activity, but these have yet to be established [Kowaltowski et al., 1998; Pedrajas et al., 1999]. Some investigators have reported that Prx has

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an anti-apoptotic affect in vitro [Zhang et al., 1997]. In addition an in vitro study indicated that the proliferative effect of Prx is possibly associated with the role of H_2O_2 as a secondary messenger [Zhang et al., 1997].

To investigate the physiologic relevance of the Prx I and II expressions induced by H_2O_2, we compared the survivals of MCF10A normal breast cell line and MCF-7 breast cancer cell line following exposure to H_2O_2. Then we induced the transient overexpression of Prx I and II in MCF10A cells by transfection to demonstrate whether Prx I and II are able to protect cells from the cell death induced by H_2O_2.

MATERIALS AND METHODS

Materials

PMSF, leupeptin, aprotinin, Triton X-100, DTT, HEPES, and diethylpyrocarbonate (DEPC) were obtained from Sigma, Trizol reagent from Gibco BRL, the First strand cDNA synthesis kit from Boehringer Mannheim, *Taq* DNA polymerase from TaKaRa Shuzo Co, antibody against prx isozymes (I, II, III, IV, and V) were kindly provided by Ho-Zoon Chae (Chungnam University, Daejeon, Korea). Normal breast tissue sample and cancer breast tissue sample were obtained from randomly breast cancer patients at Seoul National University Hospital. Horseradish peroxidase-conjugated anti-rabbit whole IgG was purchased from Jackson ImmunoResearch Laboratories, Inc., and the Enhanced Chemiluminescence Detection (ECL) system from Amersham Corp. Protein concentrations were determined using a protein assay kit from Pierce. The annexin V-FITC apoptosis detection kit was obtained from BD PharMingen.

Cell Culture

The MCF-7 and MCF10A cell lines were obtained from the Korean Cell Line Bank. MCF-7 was cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), and 100 units of penicillin, and 100 μg of streptomycin/ml in a 37°C incubator under 5% CO_2. MCF10A was cultured in Dulbecco's modified Eagle's medium (DMEM): Ham's F-12 supplemented with 5% horse serum, human insulin 10 μg/ml, epidermal growth factor (EGF) 10 μg/ml, cholera toxin (100 ng/ml), hydrocortisone 0.5 ng/ml, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate at 37°C in a humidified atmosphere consisting of 95% air and 5% CO_2.

RNA Isolation and RT-PCR

Total RNA was isolated from H_2O_2-treated or control MCF-7 cells using TRIzol reagent. The RNA pellets obtained were dissolved in diethylpyrocarbonate (DEPC)-treated H_2O at concentrations of 0.5 μg/μl to 1.0 μg/μl and then stored at −70°C. The quantity and quality of the RNA preparations were determined by measuring absorbance at 260 nm and 280 nm. One microgram of total RNA was reverse transcribed using a first strand cDNA synthesis kit with random primer $p(dN)_6$, according to the manufacture's instructions. Forward primers based on the conserved amino acid region were designed to include the *Hind*III site, and the initiation codon and reverse primers included an *Xba*I site and the stop codon. Their sequences were as follows: forward primers 5′GGGGCCAAGCTTATGTCTTCAGGAAATGCTAA3′ for Prx I and 5′TTCAAAAAGCTTATGGCCTCCGGTAACGCGCG3′ for Prx II.; reverse primers 5′CGGGCCTCTAGATCACTTCTGCTTGGAGAAAT3′ for Prx I and 5′GGCCCCTCTAGACTAATTGTGTTTGGAGAAA3′ for Prx II. The amplification conditions used were 35 cycles of: −94°C for 30 s, 65°C for 1 min, and 72°C for 1 min for Prx I, and 35 cycles of: −94°C for 30 s, 58°C for 1 min, and 72°C for 1 min for Prx II. PCR products were subcloned into pcDNA6B basic vector (Invitrogen) to yield pcDNA6B-PrxI and pcDNA6B-PrxII. Clones containing the coding sequences in the correct orientation were selected and used for transfection.

Transfection of Eukaryotic Cells

MCF10A cells were plated at a density of 3×10^5 cells/ml per 60 mm dish and allowed to recover for 24 h. They were then incubated with 4 μg of the appropriate DNA and 20 μl of LipofectAMINE^{TM} in 3 ml of opti-MEM (Life Technologies, Inc.) for 6 h, after which 3 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum was added to the transfection mixture and cells were then incubated for an additional 18 h. The efficacy and reproducibility of transfection were confirmed by immunoblot analyses of *Prx*I and *Prx* II protein in each batch of cells.

Immunoblot Analysis

H_2O_2-treated cells or control MCF-7 cells were homogenized in 20 mM HEPES (pH 7.2); 150 mM NaCl; 1% Triton X-100; 2% cholic acid; 1 mM EDTA; 1 mM EGTA; 0.1 mM DTT, 10 μg/ml leupeptin; 10 μg/ml aprotinin; and 1 mM phenylmethylsulfonyl fluoride (PMSF). These homogenates were agitated for 20 min at 4°C and centrifuged at 13,000g for 20 min at 4°C. The pellet was discarded and the supernatant containing the protein was transferred to a clean tube. Total protein concentration was determined using the micro-bicinchoninic acid (BCA) protein assay (Pierce, Rochford, IL) according to the manufacturer's instructions. Samples each containing 30 mg of protein, along with molecular weight marker (SeeBlue TM Prestained standards, Novex) and a positive control for the antibody (Jurkat cell lysate, Upstate Biotechnology, Lake Placid, NY), were subjected to 10% polyacrylamide gel electrophoresis under reducing conditions, and the proteins were transferred to a nitrocellulose membrane (Sigma Chemical Company, St. Louis). After nonspecific sites were blocked with 5% powdered milk in 0.05% Triton X-100/Tris-buffered saline (TBS-T) for 1 h, blots were incubated overnight with an IgG-purified rabbit polyclonal Prx antibody in a solution containing 5% powdered milk and 0.05% Triton X-100/TBS. The blots were then washed three times in TBS-T for 10 min each and incubated with a peroxidase-conjugated goat anti-rabbit IgG at a concentration of 1 μg/ml in 5% powdered milk in 0.05% TBS-T. All samples were also blotted for β-actin (Clone AC-15, Sigma-Aldrich, Ireland Ltd, Dublin, Ireland) to normalize protein amounts (as not shown).

Annexin V-FITC Apoptosis Detection

H_2O_2-treated or control MCF10A and MCF-7 cells were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml. These mixtures (100 μl) were then transferred to a 5 ml culture tube and 5 μl of Fluorescein-conjugated Annexin V (Annexin V-FITC) and 5 μl of Propidium iodide (PI) were added. The cells were gently vortexed and incubated for 15 min at room temperature in the dark, and 400 μl of binding buffer was added to each tube. Flow cytometry was then performed within 1 h.

Cell Viability Assay With Trypan Blue

We placed 0.5 ml of a suitable cell suspension (dilute cells in complete medium without serum to an approximate concentration of 1×105 to 2×105 cells per ml) in a screw cap test tube and added 0.1 ml of 0.4% Trypan Blue Stain, mixed thoroughly.

After it allowed to stand 5 min at 15 to 30°C (room temperature), fill a hemocytometer as for cell counting. Under a microscope, observe if non-viable are stained and viable cells excluded the stain.

RESULTS

H_2O_2 acts in a concentration-dependent manner to induce cell death in a variety of cell types [Ichimiya, 1997; Hampton and Orrenius, 1998]. To examine the effect of H_2O_2 treatment on Prx expression, MCF-7 cells were exposed to various doses of H_2O_2 for 24 h. Using five isoform-specific antibodies, we investigated the presence of Prx I, II, III, IV, and V by immunoblot analysis in the H_2O_2-treated MCF-7 cells. As shown in Figure 1, H_2O_2 treatment induced Prx expression in a dose-dependent manner. Significant reductions in Prx I and II were detected at H_2O_2 concentrations above 600 μM. Western blotting of Prx III, IV, and V showed no dose-dependent reduction in expression in MCF-7. The expressions of Prx I and II mRNA as determined by RT-PCR showed similar amount of dose-dependent reductions.

Trypan blue exclusion was used to assess time-dependent cell viability after H_2O_2 treatment. As shown in Figure 2, treatment with H_2O_2 also resulted in cell death in a time-dependent manner. H_2O_2 was found to induce cell death in both MCF-7 and MCF10A normal breast cell lines by annexin V assay. H_2O_2 treatment (800 μM) decreased cell alive by 39.1% in MCF10A cells and 11.9% in MCF-7 cells (Fig. 3).

We also examined expressions of Prx I and II in normal human breast tissues, cancer tissues, and in MCF10A and MCF-7. Prx I and II were detected in all samples by RT-PCR and also by immunoblotting. Prx I and II were overexpressed in human breast cancer tissues and MCF-7 cells compared to normal human breast tissues and MCF10A cells, respectively (Fig. 4).

To investigate whether Prx I and II are able to protect cells from the cell death induced by H_2O_2, we induced the transient overexpression

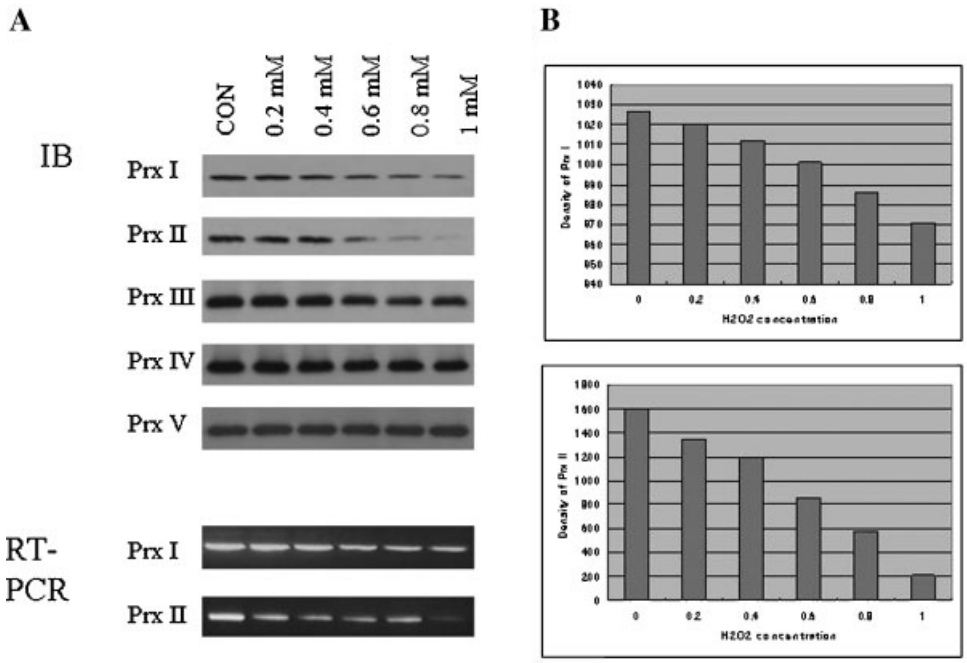

Fig. 1. Detection of Prx isozyme proteins in H_2O_2-treated MCF-7 cells by immunoblotting and RT-PCR. **A**: Expression of Prx by H_2O_2 was found to be concentration dependent. MCF-7 cells were exposed to the indicated concentrations of H_2O_2 for 24 h. Western blotting and RT-PCR analysis were performed as described in Materials and Methods. Western blotting was employed to assess the levels of the Prx isozymes (I, II, III, IV, and V) using Prx isozyme-specific antibodies. The same membranes were stripped and re-blotted with Prx isozyme-specific antibodies to demonstrate equal protein loadings. Results show one of three representative experiments. **B**: Densitometry measurements from three time course experiments demonstrating a dose-related decrease in Prx I and II levels (data presented as mean ± SE, $P < 0.001$ compared with control value).

of Prx I and II in MCF10A cells by transfection. In response to stimulation by 800 μM H_2O_2, cell survival was decreased by 29% in MCF10A transfected with vector alone and 3.6% in cells with Prx I and II transfection (Fig. 5).

DISCUSSION

In mammalian cells, a variety of extracellular stimuli, including tumor necrosis factor-α, FGF [Lo and Cruz, 1995], PDGF [Shibanuma et al.,

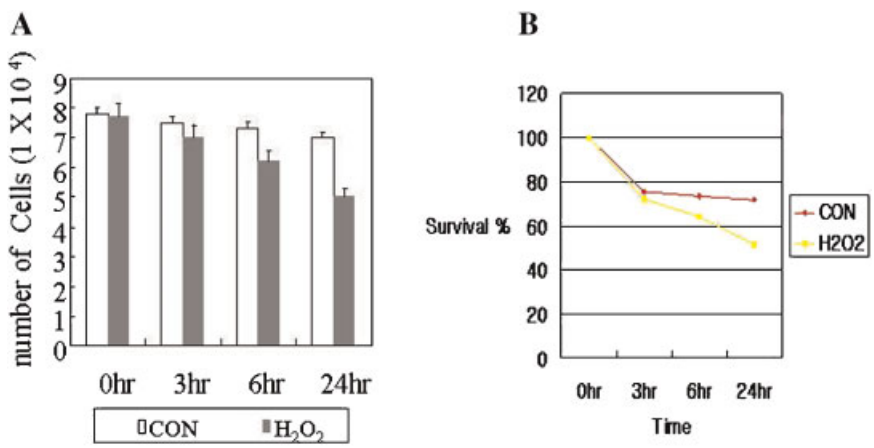

Fig. 2. The cell viability of MCF-7 cells treated with time-dependent H_2O_2. **A**: MCF-7 cells were exposed to 800 μM H_2O_2 for 3, 6, and 24 h and surviving cells (those able to exclude trypan blue) were counted. **B**: A data appeared percentage survival. Data are reported as percent survivals versus the control (untreated) cells and. Data represent the means of at least three independent experiments and standard errors of the means [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

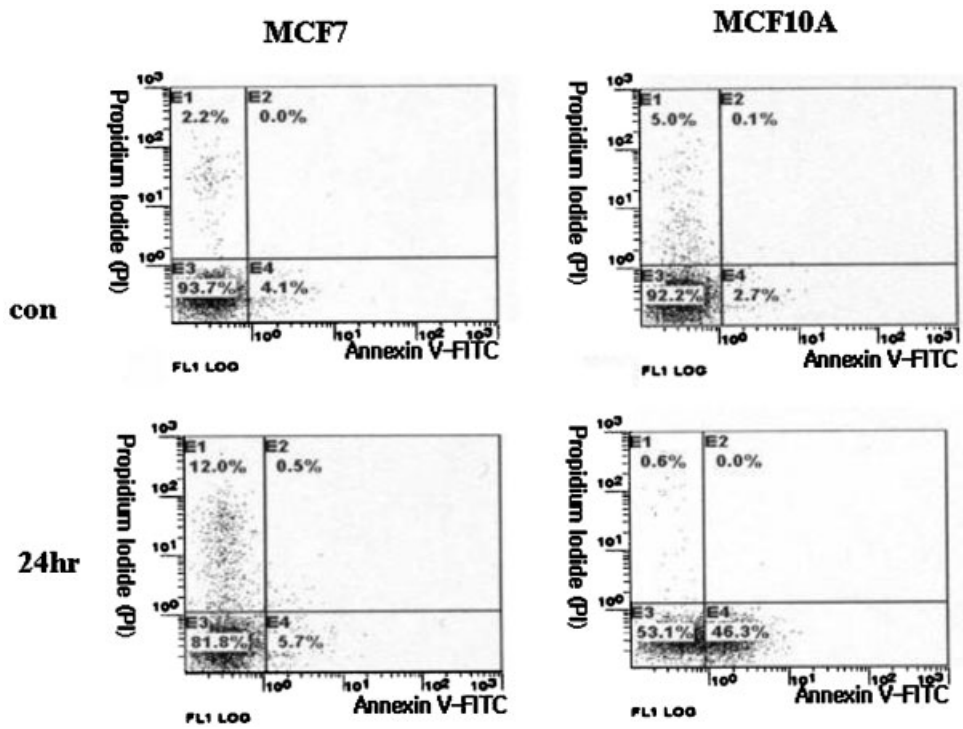

Fig. 3. H_2O_2-induced cell death in MCF10A and MCF-7 cell lines. MCF10A and MCF-7 cells were left untreated or treated for 24 h with 800 μM of H_2O_2. Cells were stained with fluorescein isothiocyanate-conjugated annexin V in a buffer containing propidium iodide and analyzed by flow cytometry. For each group of cells, the percentage of early apoptotic cells is shown in the lower right quadrant. Cells in the later stages of apoptosis had compromised membranes and stained for both annexin V and propidium iodide, and are shown in the top left quadrant. Dead cells only incorporated propidium iodide (top right). The lower left quadrant contains the vital population. Results show one of three representative experiments.

1991; Ushio-Fukai et al., 1998], and EGF [Bae et al., 1997; Factor et al., 1998; Nagayasu et al., 1998; Barrett et al., 1999] induce transient increase in the intracellular concentration of H_2O_2. The transient nature of receptor-mediated intracellular H_2O_2 increase suggests that, in addition to its production, the rapid removal of H_2O_2 is important for receptor signaling. Oxidative stress may be broadly defined as an imbalance between oxidant production and the anti-oxidizing capacity of a cell [Cross et al., 1987; Halliwel et al., 1992]. Yet the relationship between oxidative stress and the pathobiology of diseases is unclear, largely due to a lack of understanding of the mechanisms by which ROS functions in the normal and diseased state.

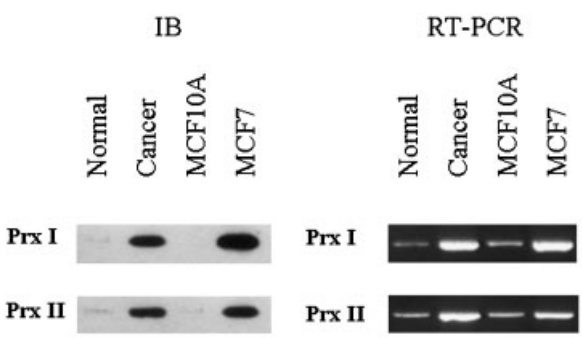

Fig. 4. Expressions of Prx I and II in normal human breast and breast cancer tissues, and in MCF10A and MCF-7 cell lines by immunoblotting and RT-PCR. Results show one of three representative experiments.

Together with glutathione peroxidase and catalase, peroxiredoxin (which reduces H_2O_2 to H_2O) enzymes play an important role in eliminating peroxides generated during metabolism. In our previous report, we demonstrated the Prx overexpression in human breast cancer tissues [Noh et al., 2001]. However, we could not find any significant association between Prx overexpression and clinical parameters such as tumor size, lymphatic invasiveness, hormone receptor status or nuclear and histologic grade in human breast cancer tissues.

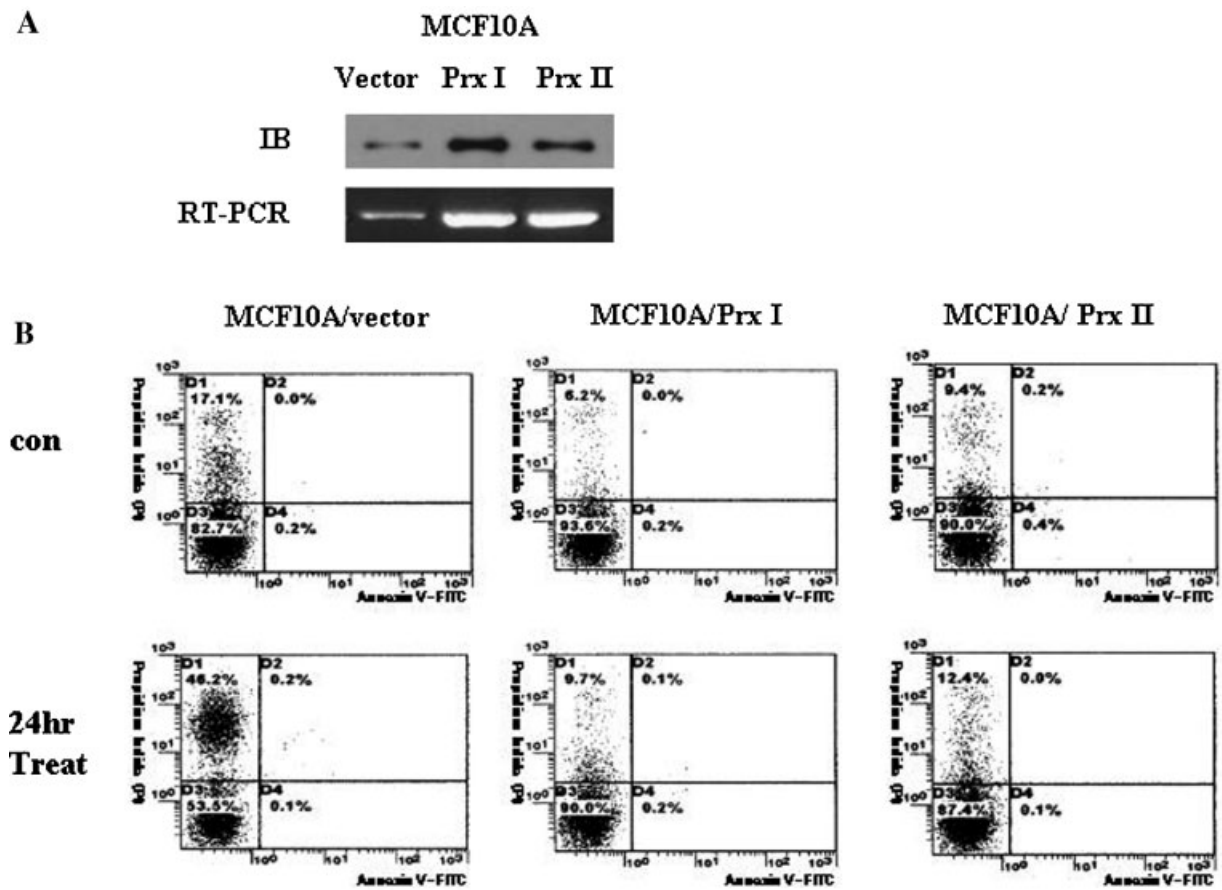

Fig. 5. H_2O_2-induced cell death in Prx I-transfected MCF10A cells. **A**: Expressions of Prx I and II in vector-transfected MCF10A, and in Prx I-transfected MCF10A cells by immunoblot analysis and RT-PCR. **B**: Vector transfected MCF10A and Prx I transfected MCF10A cells were left untreated or were treated for 24 h with 800 μM H_2O_2. Cells were stained with fluorescein isothiocyanate-conjugated annexin V in a buffer containing propidium iodide and analyzed by flow cytometry. Results show one of three representative experiments.

Apoptosis can be triggered by a variety of signals and pathophysiologic conditions, which include oxidative stress. Moreover, the inhibition of apoptosis can cause abnormal cellular proliferation and may be related to cancer development. Endogenously produced reactive oxygen intermediates (ROI) and H_2O_2 are known to act as intermediates in apoptotic signaling, based on the finding that ROI generation is detected in cells undergoing apoptosis. Moreover, the exposure of cells to low concentration of exogenous ROI, such as H_2O_2, also induces apoptotic cell death, whereas higher amounts can cause necrosis [Dypbukt et al., 1994].

Prx I and II, which are abundant in cytosol, may play an important role in eliminating the H_2O_2 generated as a by-product of metabolism in the cytosol [Kang et al., 1998]. Zhang et al. [1997] reported the results of a cell transfection study that linked Prx II and anti-apoptotic activity. Prx was suggested to be an anti-apoptotic factor that inhibits poly ADP-ribosyl polymerase (PARP) cleavage triggered by diverse stimuli, such as serum deprivation or etoposide or ceramide treatment [Lazebnik et al., 1994]. Kang et al. [1998] reported that Prx I and II might participate in the signaling cascades of growth factors and tumor necrosis factor-α by regulating the intracellular concentration of H_2O_2. They also found that Prx II is able to inhibit the release of cytochrome-c from mitochondria to the cytosol, thus preventing cellular hydrogen peroxide accumulation.

In the present study, the viabilities of MCF10A and MCF-7 were severely compromised as the H_2O_2 concentration increased. However, transfection with Prx I and II enabled MCF10A cells to resist H_2O_2 induced cell death. These findings suggest that Prx I and II, which are overexpressed in breast cancer cells, may have important functions as inhibitors of cell death during cellular response to oxidative stress.

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