The Effects of Peroxiredoxin Ⅲ on Human HeLa Cell Proliferation

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ABSTRACT

Background: Peroxidases (Prx) of the peroxiredoxin family reduce hydrogen peroxide and alkyl hydroperoxides to water and alcohol respectively. Hydrogen peroxide is implicated as an intracellular messenger in various cellular responses such as proliferation and differentiation. And Prx I activity is regulated by Cdc-2 mediated phosphorylation. This work was undertaken to investigate the proliferation role of peroxiredoxin III as a member of Prx family in Prx III overexpressed HeLa cell line. **Methods:** To provide further evidence of proliferation, we selected Prx III stably expressed HeLa Tet-off cell lines. Cell proliferation was examined by using proliferation reagent WST-1 in the presence or absence of doxycycline. Prx III, 2-cys Prx enzymes exist as homodimer. The activation of Prx III heterodimer with induced and endogenous Prx III was examined by immunoprecipitation. **Results:** Immunoprecipitation analysis of the induced and endogenous Prx III with anti-myc showed that the induced wild type (WT) and dominant negative (DN) Prx III from HeLa Prx III Tet-off stable cell heterodimerized with endogenous Prx III each other. And the expression level of induced Prx III was examined after addition of doxycycline. By 72 hr, the expression level of induced Prx III was diminished gradually and the half-life of the induced wild type Prx III was approximately 17 hr. The proliferation experiment demonstrated that the relative proliferation value of induced and endogenous WT Prx III stable cell has no changes but the DN Prx III induced HeLa Tet-off stable cells were lower than endogenous Prx III. **Conclusion:** In conclusion, the HeLa dominant negative Prx III Tet-off stable cells were decreased the proliferation. **(Immune Network 2003;3(4):276-280)**

Key Words: Peroxiredoxin III, protein half life, proliferation, WST-1, inducible dominant negative mutant

Introduction

Although H_2O_2 generally considered a toxic by-product of respiration, recent evidence suggests that the transient generation of H_2O_2 is an important signaling event triggered by activation of many cell surface receptors (1-4). It has thus been proposed that H_2O_2 is an intracellular messenger that mediates various cellular functions, including the activation of transcription factors such as NF-$_k$B (5) and AP-1 (6,7) as well as the inactivation of protein-tyrosine phosphatases (8-12). Members of the Prx family of peroxidases are present in organisms from all kingdoms (13,14). Members of the Prx family can thus be divided into two subgroups as follows: 2-Cys Prx proteins, which contain both the NH_2- and COOH-terminal Cys residues, and 1-Cys Prx proteins, which contain only the NH_2-terminal Cys (15).

Prx isoforms are distributed differentially within cells: Prx I and II are localized to the cytosol (16, 17); Prx III is synthesized with a mitochondrial-targeting sequence and is restricted to mitochondria; Prx IV is synthesized with an NH_2-terminal signal sequence for secretion and is present in the endoplasmic reticulum as well as in the extracellular space; Prx Ⅴ is expressed in long and short forms that are located in mitochondria and peroxisomes, respectively (18); and Prx VI is found in the cytosol (19,20). When overexpressed in various cell types, Prx enzymes efficiently reduced the increase in the intracellular concentration of H_2O_2 induced by platelet-derived growth factor or tumor necrosis factor-α, inhibited

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the activation of NF-$_k$B induced by tumor necrosis factor-α, and blocked apoptosis induced by ceramide (19,21,22), suggesting that they function in signaling cascade by removing H_2O_2. Purified Prx I is phosphorylated on Thr90 by cycline-dependent kinase (CDKs), resulting in a marked inhibitionof theperoxidase activity of Prx I. From these Prx I inactivation, the accumulated intracellular H_2O_2 might take part in cell cycle progression (23). In this paper, we demonstrated that mitochondrial form inducible Prx III-WT HeLa stable cell showed no difference with endogenous Prx III HeLa cell in proliferation. But the inducible Prx III-DN HeLa cell showed apparent decrease in proliferation. These results suggested that the nonfunctional, dominant negative Prx III is responsible for H_2O_2 increase in HeLa cells and that the accumulated intracellular H_2O_2 might be a role in the HeLa cell proliferation in the mitochondria.

Materials and Methods

Materials. Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, G418 and trypsin-EDTA were obtained from Life Technologies Inc.; HeLa Tet-off cells, pTRE vector, pTK-Hyg plasmid, Tet-system approved fetal bovine serum (FBS) were from Clontech; Superfect was from Qiagen; Doxycyclin was from Sigma. Antibody sources were as follows: Santa Cruz; anti-phosphotyrosine (4G10), anti-tubulin (B-5-1-2), Sigma. Rabbit polyclonal antibody to Prx III was prepared as described (19).

Plasmid constructs. Prx III (D49396) was amplified from a human liver cDNA library by PCR and subcloned into pShooter vector (Invitrogen) at Pst I and Not I restriction site. In order to construct myc-epitope-tagged Prx III, we made the forward primer (5'-TTAATAGGATCCAACCCACTGCTTACTGG CA-3') and the reverse primer for an myc epitope sequence at C-termini (5'-AGTATAGGATCCGCT-GATC AGCGAGCTTCTAG-5') and ran PCR with pShooter-Prx III as template. The PCR product directly ligated into pTRE vector (Clontech) at BamH I restriction site, named as pTRE-III-WT. The identity of all PCR products was confirmed by nucleotide sequencing (NCI-Frederick Cancer Research & Development Center). A Serine mutation at Cys108 (C108S) of Prx III was made by standard PCR-mediated site-directed mutagenesis with pTRE-Prx III as template and complementary primers containing a single-base mismatch that converts the codon for Cys to one for Ser. The C108S mutant of Prx III subcloned into pTRE vector was used as template for second round of mutagenesis to yield the Cys108 and 229 double mutant named as pTRE-III-DN.

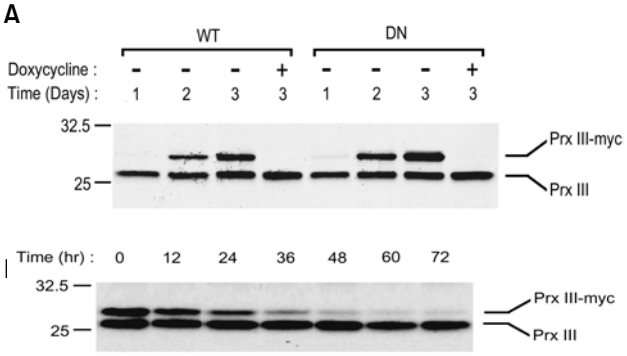
Cell culture, transfection and stable cell lines. Human HeLa Tet-off cells (Clontech) were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 U/ml) and G418 (100μg/ml) at 37°C in 5% CO_2 incubator. Cells were plated at a density of 2×10^5 per 60 mm dish and allowed to recover for 24 h. They were then co-transfected with 5μg of pTRE-III-WT or pTRE-III-DN, 0.5 g pTK-Hyg (Clontech) and 30μl Superfect reagent (Qiagen) in 1.35 ml serum free DMEM. After 2.5 hr, 3 ml of DMEM containing 10% FBS, doxycycline (1μg/ml, sigma) and G418 (100μg/ml) were added to the trans-fection mixture, and the cell were incubated for an additional 24 hr. After incubation, the cells were transferred to 150 mm culture dish to select hygromycin-resistant clones after further incubation. The media containing 10% Tet-system approved FBS, doxycycline, G418 and hygromycin (200μg/ml).

Immunoprecipitation and immunoblot analysis. HeLa cells were washed once with ice-cold phosphate-buffered saline, and lysed at 4°C for 20 min in lysis buffer containing 25 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EGTA, 1 mM dithiothreitol, 25 mM glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10μg/ml leupeptin. Cleared lysates were obtained by centrifugation at 12,000 g for 10 min and the protein concentration was measured by Bradford assay. For immunoprecipitations, cleared lysates (1,000μg in 1 ml) were precleared with protein G-sepharose beads (Amersham Pharmacia Biotech) for 1 hr at 4°C. 1μg of anti-myc monoclonal antibody was added and incubated overnight at 4°C with rotating. Protein G-sepharose was added and incubated at 4°C for 1 hr. Immunoprecipitates were washed four times with 1 ml of ice-cold lysis buffer, resuspended in 2 Laemmli sample buffer, and processed for immunoblotting. For immunoblot assay, samples were resolved by 12% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with an appropriate antibody.

Cell proliferation assay. We used the cell proliferation reagent WST-1 (4-3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio-1.3-benzenedisulfonate sodium salt, Roche) for assay of cell proliferation. Cells were resuspended in DMEM medium in the presence or absence of doxycycline and seeded in 96 well Falcon 3,072 plates (Becton Dickinson, Lincoln Park, NJ). 10 μl of WST-1 solution was added to 100μl of PBS buffer per well and the cells were then incubated for 1 hr. The plates were read on a Molecular Devices Thermo max Microplate reader, using a test wavelength of 450 nm, a reference wavelength of 595 nm.

Results

Expression of PrxIII in HeLa cells under the control of tetracycline. To investigate a physiological role of Prx III, we established stable expression of myc-epitope tagged wild (pTRE-III-WT) and cysteine mutant Prx III (pTRE-III-DN), where two conserved and active site cystein residue was replaced by serine residue. We cotransfected pTRE-III-WT or pTRE-III-DN. After selection, 49 Prx III WT and 36 DN surviving colonies were screened by the western blotting with Prx III antibody, each 8 and 10 colonies were selected. Based on expression level, we chose a clone of HeLa cells. For screening the elevated levels of myc-tagged wild and mutant Prx III proteins, we induced expressions of Prx III-WT-Myc and Prx III-DN-myc by removal of doxycycline. After 3 days, the expression levels of each type of cells were similar to those of endogenous Prx III, respectively (Fig. 1A). Next question is how long the induced protein is lasting in the cells. So we quantitatively analyze the degradation of induced Prx III after the addition of doxycycline. By 72 hr, the expression level of induced Prx III was diminished gradually (Fig. 1B). From quantitative analysis, the half-life of induced Prx III was approximately 17 hr (Fig. 1C).



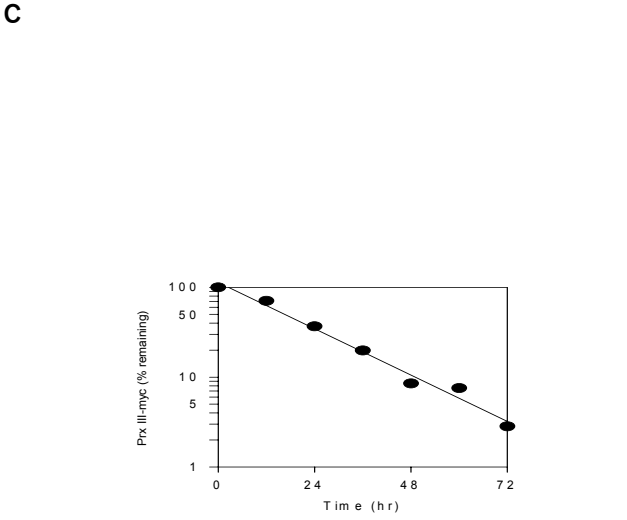


Figure 1. Expression of wild and mutant Prx III in HeLa cells after removal of doxycycline. A. HeLa Tet-off cells were stably transfected with wild type (WT) and mutant type (DN) of Prx III, respectively. Cells were cultured in either the presence (+) or absence (-) of doxycycline (1μg/ml) for indicated time points. Analysis of induced Prx III degradation. HeLa Tet-off cells were stably transfected with wild type (WT) Prx III. To induce an expression of Prx III, cells were cultured in the absence of doxycycline for 48 hr. After doxycycline (1μg/ml) was added into culture media, cells were further cultured for the indicated time points. B, lysates (5μg proteins) were resolved by 12% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-Prx III antibody. C, chemiluminescence signals developed and collected in Kodak Image Station and quantitated by using Image-Quant software, according to manufacturer's instruction. Molecular size standards in kilodaltons are shown on the left.

Proliferation. To investigate how mitochondrial Prx III related to cell growth in the cells, Prx III DNA was stably transfected into HeLa cells. Tetrazolium salt has been used to develop a quantitative colorimetric assay for cell survival. The assay detects living, but not dead cells and the signal generated is depend on the degree of activation of the cells. For this purpose, we used MTT derivative WST-1, which is indicator of mitochondrial dehydrogenase in viable cell. A role in normal growth was demonstrated in HeLa cells by using human Prx III, resulted in a no difference in serum-dependent growth (Fig. 2A). But dominant negative stable cell line resulted in a decrease (Fig. 2B).

Discussion

Subcellular distribution of Prx III is in mitochondria. The 2-Cys Prx III enzymes exist as homodimers with the two monomers oriented in a head-to-tail manner (13,15). To investigate a physiological role of Prx III, we established stable expression of myc-epitope tagged wild and cystein mutant dominant negative in HeLa Tet-off cells. But Prx III dimerization is required for the activation in Prx III inducible stable cells. The expressed mutant Prx III can form heterodimer with endogenous proteins (data not shown). So we use this cell line for further Prx III physiological study.

Moreover expressed inducible Prx III is maintained for about 72 hr after the addition of the doxycycline. Even the half-life of Prx III is 17 hr. This results shows the stability of inducible Prx III is enough to examine the cell proliferation for 3 days. After 17hrs expression of Prx III inducible stable cells, at least the 50% of the induced Prx III form the dominant negative heterodimer and works as a dominant ne-

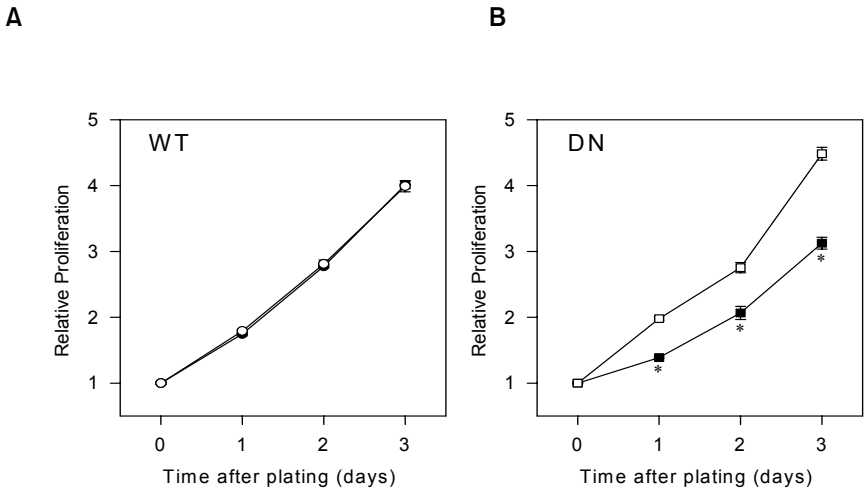


Figure 2. Role of Prx III in HeLa Tet-off cell proliferation. HeLa Tet-off cells were stably transfected with wild type (WT) in and mutant type (DN) of Prx III, respectively. Cells were seeded onto 96-well plate (1×10^4 cells per well) and cultured in either the presence (white) or absence (black) of doxycycline (1 ㎍/ml) for indicated time points. After washing with PBS buffer, cell proliferation was quantified using cell proliferation reagent WST-1 (Roche). A, Prx III WT and B, Prx III-DN stable cell. Values are the mean±S.E.M. (n=8). *, $P<0.001$, significant difference between HeLa cells in the presence and absence of doxycycline.

gative mutants.

In the experiment of the mitochondrial Prx III relation to cell growth in the cells, we used Prx III stable cell with wild type and dominant negative type, respectively.

Induced Prx III WT protein of stable cell line had no effect in the cell growth. From this result, endogenous Prx III protein of Prx III WT stable cell line might be enough amounts to functioning in mitochondria. Base on unique Prx structural feature, cystein double mutant of Prx where two conserved, active site cystein residues are replaced by serine residues, served as a dominant negative mutant to titrate out the endogenous wild-type proteins. And dominant negative Prx III protein has no more activity as a peroxidase. Mitochondria are a major physiological source of reactive oxygen species (ROS), which can be generated during mitochondrial respiration (24). Superoxide radicals, formed by mitochondrial electron transport chain or by an NADH-independent enzyme, can be converted to H_2O_2 and to the hydroxyl radical (25).

Thus mitochondria are continually exposed to ROS that cause peroxidation of membrane lipids, cleavage of mitochondrial DNA, and impairment of ATP generation, with resultant irreversible damage to mitochondria.

Arai et al. shows that mitochondrial PHGPx (phospholipids hydroperoxide glutathione peroxidase) was prevent cell death that was caused by ROS generated in mitochondria and by exogenously added hydroperoxides (26). The result of dominant negative Prx III stable cell reflects that generated ROS in mitochondria causes cell death.

Prx V, the member of the Prx family, is localized intracellulary to cytosol, mitochondria and peroxisomes. The mitochondrial fraction of Prx V has the possibility to affect Prx III overexpressed HeLa Tet-off cell proliferation. Seo et al. showed the amounts of various Prx isoforms in mammalian cells (16). In HeLa cells, the amount of Prx III and V was similar and relatively smaller than other isoforms. Moreover, PrxIII was mitochondrial dominant form, but one third of total Prx V was mitochondrial form. Prx V could not overcome the effect of Prx III in Prx III overexpressed HeLa Tet-off cell. Therefore, our result suggest that Prx III in mitochondria has an important effect in cell growth.

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