Involvement of Peroxiredoxin I in Protecting Cells from Radiation-Induced Death

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Peroxiredoxin I/Ionizing radiation/RNA interference.

Peroxiredoxin I (Prx-I), a key member of the peroxiredoxin family, reduces peroxides and equivalents through the thioredoxin system. Our previous work has shown that expression of Prx-I in mammalian cells increases following ionizing radiation (IR), indicating that Prx-I actively responds to IR-induced reactive oxygen species (ROS) and suggesting that Prx-I plays an important role in protecting cells from IR-induced death. To test this hypothesis, we suppressed the expression of Prx-I in SW480 cells by RNA interference. Our results show that IR induces the expression of Prx-I in SW480 cells in a dose- and time-dependent manner. The recombinant siRNA vector targeting Prx-I dramatically reduced the expression of Prx-I in SW480 cells. When Prx-I was knocked down in SW480 cells, the cells exhibited a decreased growth rate, a reduced antioxidant capability following IR and became more sensitive to IR-induced apoptosis. Together, our results demonstrate that Prx-I plays an important role in protecting cells from IR-induced cell death, which might be through scavenging IR-induced ROS in the cells.

INTRODUCTION

Ionizing radiation (IR) promotes many important cellular processes such as DNA damage, apoptosis, signal transduction, and oxidative stress. One of the early effects of IR is that it produces reactive oxygen species (ROS) such as O\(_2^-\), and H\(_2\)O\(_2\). Oxidative stress induced by IR produces a variety of highly reactive free radicals that damage cells, initiate signal transduction pathways, and alter gene expression. ROS can induce the cellular antioxidant defense enzymes such as superoxide dismutase, and glutathione peroxidase. For example, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase were activated up to 5-fold in RRC cells (from U251 human glioblastoma cell line), compared to the parent cells after radiation. The relationship between IR and antioxidant enzymes has been well investigated. Antioxidant enzymes can attenuate the radiation injuries.

Recently, a new family of highly conserved antioxidant enzymes peroxiredoxin (Prx), has been discovered and shown to play a critical role in peroxide detoxification with reducing equivalents provided through the thioredoxin system but not from glutaredoxin. All Prx proteins contain a conserved cysteine residue in the N-terminal region that is the active site of catalysis. There are six isoforms of peroxiredoxin in mammalian cells, which can be divided into two subgroups based on conservative cysteine residues: one group in which two cysteine residues are conserved (Prx-I~IV, V), and the other group in which one cysteine residue is conserved (Prx-VI). Prx-I and II are very similar to each other, and both genes are involved in redox regulation of the cell and reduce peroxides. But Prx-I protein has homology to proliferation-associated gene product, while the other one has homology to thiol-specific antioxidant proteins.

Peroxiredoxin isoforms are distributed differently within cells. Prx-I is found in abundance in the cytoplasm as homodimers. The antioxidant molecular mechanism of its homologue Prx-II has been elucidated by several groups based on its molecular structure. From the results of biochemical and physiological studies, Prx-I has been implicated in a number of cellular functions, such as cell proliferation and differentiation, enhancement of the natural killer cell activity, and intracellular signaling, in addition to the antioxidant activity. Increased Prx-I expression has been observed in various kinds of cancer cells, indicating it may be involved in cancer development or progression.
Our previous work has demonstrated that the expression of Prx-I was up-regulated by IR in mouse intestinal epithelia and cultured IEC-6 cells. This was consistent with studies from other groups. It was found that IR induced Prx-I protein and mRNA expression in both human HT29 colon cancer and rat C6 glioma cells in a dose- and time-dependent manner. The questions are whether the induction of Prx-I by IR is a common phenomenon, and whether Prx-I plays an important role in protection against IR. In this study, we sought to inhibit the expression of Prx-I in SW480 cell line by RNA interference, and evaluate its effect on IR-induced cell death.

**MATERIALS AND METHODS**

**Materials**

SW480 cell line was obtained from Shanghai Cell Bank (http://www.ctcccas.ac.cn/xibao). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were purchased from Hyclone (Logan, USA). Antibodies were from Santa Cruz (California, USA). Plasmid pAVU6+27 was a gift from Prof. Engelke DR (Department of Biological Chemistry, University of Michigan, USA). Oligonucleotides were synthesized by Shanghai Shenyou Shengwu Jishu Corporation (Shanghai, China). Malondialdehyde (MDA) determination kit and total antioxidant test kit were from Institute of Nanjing Jiancheng Shengwu Gongcheng (Nanjing, China, cat # A003 and A015). All other chemicals were of the highest grades available commercially.

**Cell culture and irradiation**

SW480 cells from a human colon adenocarcinoma were cultured in DMEM containing 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). To test the induction of Prx-I by ionizing radiation in a dose-dependent manner, the 80% confluent monolayer SW480 cells were irradiated with a 60Co source at dose of 5, 15, 25 Gy (dose rate 4.18 Gy/min). After irradiation, the medium was immediately replaced with fresh medium. The dishes were of the highest grades available commercially.

**Cell cycle determination**

Exponentially growing cells were cultivated and synchronized for 24 h in serum-free medium, and then changed with a complete medium before irradiation. Sham control and radiated cells were harvested by trypsinization at 24hr post-radiation, washed with ice-cold PBS, and stored at 4°C. Before DNA analysis, DNA content was labeled with propidium iodide (PI) in the presence of the RNase (1 mg/ml) for 30min at room temperature. The presence of apoptotic cells was detected by determination of the SubG1 population. Flow cytometry analysis was performed on a FACSscan, and data were analyzed by using Multi Cycle software (Becton Dickinson).

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**Cell growth curve**

MTT assay is based on the enzymatic reduction of the tetrazolium salt (MTT) in living, metabolically active cells. Exponentially growing cells were cultivated in 96-well plates, with 5 × 10³ cells in each well. Twelve hours later, 20 μl of MTT solution (10 mg/ml) was added into the culture media, and the plate was returned to the incubator and cultivated for another 4h. After the culture media were drained out, 200 μl of DMSO was added into each well. The plates were read on a Dynatech MR600 Microplate Reader at 540 nm. All data were normalized relative to the corresponding untreated cells.

**Protein extraction**

A cell suspension was centrifuged at 1 000 g and the cell pellet was washed in ice-cold PBS for 3 times. Cells were resuspended in cold PBS. Total proteins were extracted with cell lysis buffer containing 10mM HEPES, pH7.9, 10mM KCl, 1.5mM MgCl₂, 0.1mM EDTA, 1mM diithiothreitol, 0.5mM phenylmethylsulfonyl fluoride, and 0.6% Nonidet P-40. The extraction mixture was centrifuged at 12 000g at 4°C for 20 min. The protein concentration was determined using the Bradford dye-binding assay with bovine serum albumin as the standard. To validate the reproducibility, cell samples were repeatedly prepared at least 3 times.

**Plasmid construction**

Vectors for RNAi were constructed by modifying pAVU6+27. This vector produces dsRNA as a stem-loop structure in mammalian cells. Synthesized oligonucleotides were: 5’ TCGACGCGCACATTGCTCAGGATgctATCC-TGAGCAATGGTGCGCTTTTTTG, and 5’CTAGCC-AAAAAGCGCAACATTGCTCAGGAT cgaaATCTTGGAGAATGGTGCGGG. The sequence underlined above was corresponding to bases 641–659 of Prx-I (gi: 32455263). They were annealed and ligated to the XbaI/Sall sites of pAVU6+27. The recombinant plasmid (named as pAVU6-PrxI) was identified and prepared for cell transfection. Randomly generated oligonucleotides with the linker sequence are: 5’ TCGACCTTCTTACCTAGTTACATCATCCGTAACCTGAGGTAAGAGGT, and 5’TCTAGGC-AAAAACCTTTACCTACCTAGTTACCGaATGGGTAAGAGGG. The oligonucleotides were also annealed and ligated to pAVU6+27, using as a control for interference specificity. This control recombinant plasmid was designated as pAVU6-Con
**Cell transfection**

Exponentially growing SW480 cells were transferred to 100 mm culture dishes and cultured for 24 h to yield about 40% confluency at the time of transfection. Cell transfection using DOTAP (Roche, USA) was performed according to the manufacturers instruction. Briefly, after the cells were incubated with the mixture of DNA and DOTAP in 2 ml of serum-free medium for 4 h, the cells were washed with serum-free medium, and incubated for 24 h. Then the media were refreshed with DMEM containing G418 (200 µg/ml). After selection for 2 weeks, the bulk cultures were then used for experiments. SW480 cells were transfected with either pAVU6-Con or pAVU6-PrxI were named as SW480Con and SW480SiPrxI, respectively.

**Western Blot**

Total proteins were isolated from SW480 (with sham control and irradiated), SW480Con and SW480SiPrxI cells, and were separated on 15% SDS-polyacrylamide gel, and then transferred to nitrocellulose membranes. After being blocked for 1 h with the Tris/NaCl (50 mM Tris-HCl, 200 mM NaCl, 0.05% Tween-20, pH 7.4) containing 2% BSA, the membranes were probed with specific antibodies against Prx-I and GAPDH (Santa Cruz, cat # sc-7380 and sc-20357). Following washing, the blots were incubated for 2h with horse-radish peroxidase-labeled anti-goat IgG (Santa Cruz, cat #sc-2768). Immunoreactive bands were visualized with solution containing 1 mg/ml DAB and 0.01% H2O2. The membranes were scanned (Gel Doc 2000, Bio-Rad), and analyzed with Quantity One software (Bio-Rad Corp.). The ratio of the intensity of Prx-I / GAPDH was automatically compared.

**Clonogenic assay for cell survival**

The radiosensitivity of SW480Con and SW480SiPrxI cells was assayed by clonogenic assays. Briefly, cells in monolayer culture during the logarithmic growth phase were trypsinized and resuspended in the medium. After irradiation, cells were seeded in 6-well plates at concentrations pre-determined to give 25-200 colonies at each dose (2, 4 and 6Gy). Ten to fourteen days later, the cultures were fixed and stained with 0.5% crystal violet in absolute methanol, and colonies containing more than 50 cells were scored with a colony counter. Survival curves were generated by combining data from three independent experiments, with each experiment performed in triplicate.

**Determination of MDA concentration and total antioxidant capability**

IR induces free radicals, which damage cellular macromolecules. One of the most significant changes after radiation is that cellular lipid peroxides accumulate. Cellular lipid peroxides were estimated by measuring MDA formation with thiobarbituric acid (TBA) colorimetric analysis.\(^{(20)}\) In

![Fig. 1. Effect of radiation on Prx-I proteins in human SW480 cancer cells. A: Dose response of Prx-I. Western blot analysis was representatively performed on the cell lysate 24 hr post-irradiation with the indicated doses. The expression of Prx-I was normalized by that of GAPDH. B: Quantitation of the relative Prx-I protein level at indicated doses. Error bars indicated mean ± SD from three independent studies, *\(P < 0.05\), and \(^*\)\(P < 0.01\) vs. 0 Gy. C: Time course response of Prx-I to irradiation. Western blot analysis was performed on the cell lysate at indicated time points after 15 Gy of radiation. D: Quantitation of the relative Prx-I protein level at indicated time, *\(P < 0.05\), and \(^*\)\(P < 0.01\) vs 0 h.](https://academic.oup.com/jrr/article-abstract/46/3/305/919748)
brief, 24 h post-irradiation, cells were harvested and resuspended in lysis buffer containing 10mM HEPES, pH 7.9, 10mM KCl, 1.5mM MgCl₂, and 0.1mM EDTA and sonicated. Then 0.1 ml of the sonicated lysate was added into the solution containing TBA, and incubated at 95°C for 40 min. The absorption of supernatant was measured at 532 nm.

The total antioxidant capability (T-AOC) of SW480Con and SW480SiPrxI cells was assayed with a commercial test kit from Institute of Nanjing Jiancheng Shengwu Gongcheng (http://www.njjcbio.com/main/index.asp). All performance was carried out according to manufacturers description. Briefly, cell samples prepared as above were added into the tubes containing pholasin, then incubated at 37°C for 30 min. The cell lysate reduces Fe³⁺ to Fe²⁺, and Fe²⁺ binds with pholasin, which produces visible chelating agent. The absorption of supernatant was measured at 520 nm.

Statistical analysis
Statistical analysis was performed using the independent sample t-test. All P values were two-tailed and considered statistically significant if less than 0.05. Means, standard errors, and P values were calculated using SPSS version 9 for Windows.

RESULTS

Induction of Prx-I protein by irradiation
The effect of various doses of radiation on Prx-I protein expression was assessed in SW480 cells by immunoblotting assays (Fig. 1A–B). Results of three independent experiments showed similar dose responses. Increased expression of Prx-I was observed at lower doses, and declined expression at higher doses. Maximal induction of Prx-I occurred at 15 Gy of irradiation (1.5 fold more than normal control). Time course studies indicated that the expression of Prx-I protein increased 3 hr after exposure to a 15 Gy radiation dose, with a peak at 12 hr (2 fold more than normal control). Even after 24 hr, the expression level remained elevated (Fig. 1C–D).

Construction of SiRNA vector and its inhibition of Prx-I expression
Synthesized oligonucleotides were annealed and inserted into the XbaI and SalI site of pAVU6+27. The recombinant plasmid was screened by BamHI and HindIII digestion. Additional band (about 410bp) would appear when the recombinant plasmid was digested (Data not shown). It was then sequenced. The result showed that the inserted sequence was coincident with designed oligonucleotides. Diagram of the recombinant construct was illustrated in Fig. 2A. The recombinant plasmid of pAVU-PrxI contains a U6 promoter that directs the synthesis of oligonucleotides in an inverted repeat with 4 nt for its loop, with six T bases added at the end to serve as a termination signal for RNA polymerase III.

The recombinant and its control plasmid were transfected into cultured SW480 cells. After selection of G418 for 2 weeks, G418 (200 µg/ml) resistant cells grew very well. The protein levels of Prx-I in SW480Con and SW480SiPrxI cells were detected by Western blot. As shown in Fig. 2B, expression of Prx-I in SW480SiPrxI cells was significantly decreased, which can hardly be detected. This result showed that the expression of Prx-I protein was efficiently knocked down.

Cell growth and proliferation ability
Both SW480Con and SW480SiPrxI cells were cultivated...
in G418 containing medium at the concentration of 200 µg/ml. However, when the concentration of G418 was increased up to 800 µg/ml, SW480SiPrxI cells grew poorly compared to control, and most of these cells would die in 3 days (Data not shown).

To determine the biological function of Prx-I on cell proliferation, we first observed the cell growth of SW480Con and SW480SiPrxI cells. Cell proliferation was determined by MTT assay. As shown in Fig. 3, cell growth of SW480SiPrxI cells was significantly delayed, compared with its normal control. The proliferating time of SW480SiPrxI was prolonged to double of that in normal control. This indicates that Prx-I plays a role in cell proliferation.

Detection of cell injury after radiation

Radiation induced the expression of Prx-I in normal SW480 cells. This could be explained as adaptation of radiation. We asked whether down-regulation of Prx-I would sensitize the cells to radiation. Fig. 4 showed that cell survival of SW480SiPrxI cells was significantly decreased after radiation, compared to that of its normal control. About 30% of SW480Con cells survived after 6 Gy of irradiation, but only 6% of SW480SiPrxI cells did. This indicates radiation-induced sublethal damage is severer when the expression of Prx-I is inhibited.

Another marker of cellular injury after radiation is that lipid peroxides accumulate. This can be detected by measuring the content of MDA.22) As shown in Fig. 5, the concentration of cellular MDA increased 24h after 10Gy irradiation. However, more increase was observed in irradiated SW480SiPrxI cells than that in SW480Con cells. This result also indicated that radiation injury was severer when the expression of Prx-I was inhibited. It is apparent from these data that inhibited expression of Prx-I can enhance cell death after radiation. In other word, radiation sensitivity can be increased by the suppression of Prx-I expression.

Cell cycle arrest and increased radiation induced apoptosis

To determine the effect of IR on the cell cycle distribution, and stimulating effects of suppressed Prx-I expression on apoptotic cell death after radiation, SW480Con and SW480SiPrxI cells were harvested and analyzed by flow cytometry. Cell cycle phases of irradiated and unirradiated cells were listed in Table 1. Twenty-four hours after irradia-

| Cell type | Cell cycle distribution (%) |
|-----------|-----------------------------|
| SW480Con  | Normal | G1 | G2 | SubG1 |
| SW480SiPrxI | Normal | G1 | G2 | SubG1 |

Table 1. Cell cycle distribution of SW480Con and SW480SiPrxI after 10 Gy irradiation.

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tion, G2-M checkpoint arrest was found, and 42% of the SW480Con cell blocked in G2 phase, as 52% of SW480SiPrxI cells in G2 phase. Higher ratio of G2/G1 was observed in irradiated SW480SiPrxI cells than that in irradiated SW480Con cells. More apoptotic cell death was observed in irradiated SW480SiPrxI cells than in irradiated SW480Con cells (7.3% ± 0.9% vs. 3.4% ± 0.7%, Table 1).

Decreased total antioxidant capability by SiRNA

IR has been shown to enhance the production of cellular free radicals. Because Prx-I reduces peroxides with reducing equivalents provided through the thioredoxin system, we further asked whether enhanced cell death in SW480SiPrxI cells by radiation was due to its decreased T-AOC.

T-AOC of SW480Con and SW480SiPrxI cells was determined. As shown in Fig. 6, T-AOC in SW480SiPrxI cells was only 85% of that in SW480Con cells (P<0.05). Moreover, it decreased dramatically when SW480SiPrxI cells were irradiated. This result suggested that SW480SiPrxI cells had lower T-AOC because of inhibition of Prx-I expression, thus more ROS induced by IR remained unscavenged, and more lipid peroxides were produced. It is reasonable to deduce that Prx-I is involved in protecting cells from radiation-induced death.

![Graph showing T-AOC comparison](image)

**Fig. 6.** Total antioxidant capability in SW480Con and SW480SiPrxI cells 24 hour after 10Gy irradiation. Total antioxidant capability was significantly decreased when cells were irradiated. * P < 0.05 irradiated cells and its un-radiated counterpart. # P < 0.01 between irradiated SW480Con and irradiated SW480SiPrxI cells.

**DISCUSSION**

The novel findings in this study are that: 1) ionizing radiation induces expression of Prx-I in SW480 human cancer cell; and 2) inhibition of Prx-I expression sensitizes SW480 cell to radiation, and enhances cell death by irradiation. The cDNA of Prx-I was first cloned in 1993 applying differential hybridization technique, and it was named as MSP23, OSF-3, and PAG.** Treatment with diethylmaleate or glucose/glucose oxidase markedly enhanced the induction of Prx-I transcription, and its protein expression in the cells. Moreover, the cDNA corresponding to the thiol-specific antioxidant (TSA), which encodes a protein named as Prx, has been cloned and sequenced from yeast and mammalian cells. From the biochemical and physiological studies, Prx-I is involved in a variety of diverse cellular functions including proliferation, differentiation, and immune response. For example, Prosperi et al found Prx-I was expressed in most human cells, and it was induced to higher levels upon serum stimulation in untransformed and ras-transformed HBL100 cells. Its transcript was approximately 3-fold higher in transformed cell as compared to untransformed cells after 7–15 h of serum stimulation. In our work, we found decrease of Prx-I expression could give rise to cell growth delay, further confirming its function on cell proliferation.

It was suggested that Prx-I was a radiation responsive gene. It has been shown that Prx-I is transiently up-regulated in the irradiated testis. This result suggests that the relative radiation-resistance of Leydig and Sertoli cells can be attributed in part to the antioxidant function of the Prx system in these cells. Its highly homologous gene Prx-II was increased in tissues isolated from the patients who did not respond to radiation therapy. And treatment with a Prx-II antisense decreased the expression of Prx-II, enhancing the radiation sensitivity of cells. Moreover, it was found that IR induced Prx-I expression in both human HT29 colon cancer and rat C6 glioma cells in a dose- and time-dependent manner. In our previous work, we also found that Prx-I protein was increased in epithelial cells post radiation in vitro and in vivo by 2-dimensional electrophoresis. All these data suggested Prx-I expression could be induced in many kinds of cells. In this work, we indeed found that the expression of Prx-I in SW480 cells was also induced by IR in a dose- and time-dependent manner.

RNAi was first achieved in transient fashion by electroporation of *in vitro* synthesized dsRNA. Another more valuable approach utilizing inducible, stably maintained constructs that express stem-loop transcripts has recently been described. 33-34 The construct containing inverted sequence corresponding to target gene could efficiently transcript small RNA, thus formed hairpin structure to knock down the target gene. So target gene expression could be efficiently knocked down via this method. Our result showed the expression of Prx-I in SW480 cells was knocked down, and was hardly detected.

ROS are major mediators of irradiation damage, and numerous studies have shown that levels of redox-regulating compounds can affect cellular radiation responses. Antioxidant enzymes scavenge ROS derived from ionizing radiation. These enzymes play important roles in radiation. The newly discovered antioxidant Prx enzymes appear to contribute in this respect, although the extent of their contribution is only now being realized. Prx-I has the biochemical function of reducing peroxides. So it is suggested that Prx-
I may play a key role in radiation. As shown in our result, cellular T-AOC decreased when Prx-I expression was suppressed. Thus more ROS remained unscavenged post radiation, leading to oxidative stress, and more cell death. The relationship between Prx-I and antioxidant defense has been investigated by other researchers. Sensitization of prostate cancer cells for hydrogen peroxide or an organic hydroperoxide was observed by down regulation of Prx-I via antisense technique. Also, researchers found knockdown of peroxiredoxin I expression by RNA interference sensitized cells for APIT-induced cell death. Inhibition of its highly homologous gene Prx-II by antisense RNA, increased cell death post radiation exposure was also observed. And Prx-II was a regulator of the H2O2 signal initiated by growth factors, such as PDGF. We speculate that both genes may be involved in ROS signal pathway, regulate cell growth and scavenge IR-induced radicals. These data suggest that hydroperoxide induced stress and the modulation of peroxiredoxins might be a promising approach for tumor therapy.

The most striking finding of Prx-I expression was that it was increased in many kinds of tumor cells. Moreover, some researchers thought it might be a potential tumor marker. Prx-I was over-expressed in human breast cancer tissues compared to normal tissues. These data also suggest Prx-I is involved in tumor development or progression, and it might be a new target for tumor cells. Our current study and others suggest that Prx-I can be considered for predicting tumor response to radiation therapy and a target for rendering tumor cells more radioresponsive. It is important to elucidate the relationship between IR, Prx-I expression and carcinogenesis. This will help develop more effective radiation therapy for cancer.

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