Antisense of Human Peroxiredoxin II Enhances Radiation-induced Cell Death[1]

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ABSTRACT

Human peroxiredoxin II (Prx II) has been known to function as an antioxidant enzyme in cells. Using head-and-neck cancer cell lines, we investigated whether Prx II expression is related to the resistance of cells to radiation therapy *in vivo* and *in vitro*, and whether a Prx II antisense serves as a radiosensitizer. Increased expression of Prx II was observed in tissues isolated from the patients who did not respond to radiation therapy, whereas Prx II expression was weak in tissues from the patients with regressed tumors. Enhanced expression of Prx II in UMSCC-11A (11A) cells was also observed after treatment with γ radiation. This increased expression conferred radiation resistance to cancer cells because overexpression of Prx II protected 11A cells from radiation-induced cell death, suggesting that blocking Prx II expression could enhance radiation sensitivity. Treatment of 11A cells with a Prx II antisense decreased induction of Prx II, enhancing the radiation sensitivity. From these results, we suggest that stress-induced overexpression of Prx II increases radiation resistance via protection of cancer cells from radiation-induced oxidative cytolysis and that a Prx II antisense can be used as a radiosensitizer.

INTRODUCTION

Radiation therapy and chemotherapy in conjunction with surgical operations have been commonly used for the treatment of head and neck tumors. However, a significant number of tumors fail respond to radiation therapy and/or chemotherapy because many forms of tumors appear to become less sensitive or resistant to radiation and anticancer drugs after consecutive treatments. Although extensive studies on the molecular mechanisms of resistance to chemo- and/or radiation therapy have been carried out, problems related to overcoming this resistance remain to be solved.

The mammalian Prx[5] family is divided into two groups, based on the amino acid sequences: one group in which two cysteines are conserved (Prx I, II, III, and IV); and one group in which one cysteine is conserved (Prx V and VI; Refs. 1–3). Member of the Prx family have previously been known as thioredoxin peroxidase, which reduces H_2O_2 using the electrons provided by thioredoxin (4, 5). However, it is not yet established whether all members of the Prx family found in a variety of species actually function as a peroxidase (4–6). Some members of the Prx family do not require thioredoxin as an electron donor; therefore, they are not termed thioredoxin peroxidase (7). Mammalian tissues express Prx isoforms, and their overexpression prevented intracellular accumulation of H_2O_2, inhibiting apoptosis (7, 8).

Prx II has also been known as NKEF-B (9, 10). NKEF, M_r 48,000, is composed of two subunits linked by disulfide bonds (9). It was initially found in human RBCs and was known to play a role in enhancing cytotoxicity of natural killer cells. It was classified into two subgroups, NKEF-A and NKEF-B, which were later identified as Prx I and II, respectively; the primary sequences of Prx I and II are highly homologous to each other (10). The cDNA sequence of Prx I is homologous to a human proliferation-associated gene (PAG), and it is known to be associated with cell proliferation and differentiation (11). In contrast, the cDNA sequence of Prx II shows a homology to thiol-specific antioxidant proteins, which scavenge or suppress formation of protein thiol radicals (2). Therefore, it has been proposed that Prx II functions as an antioxidant protein that protects cells from ROS or cellular oxidative damage (3, 12).

Kim *et al.* (13) observed that induction of Prx II mRNA was increased in human endothelial (ECV304) cells treated with H_2O_2. Moreover, overexpression of Prx II in ECV304 cells exposed to *t*-butylperoxide or methyl mercury reduced ROS generation, resulting in an increased protection from oxidative stress (14). However, overexpression of Prx II did not protect the cells from oxidative damages by depletion of the intracellular GSH (13). It was therefore proposed that the activity of Prx

[5] The abbreviations used are: Prx II, peroxiredoxin II; NKEF, natural killer-enhancing factor; ROS, reactive oxygen species; GSH, glutathione; DAPI, 4′,6-diamidino-2-phenylindole; 7-AAD, 7-amino-actinomycin D; 5-FU, 5-fluorouracil.

Table 1 Profile of patients receiving chemo- or radiation therapy

Patients with head and neck tumors were divided to two groups based on their chemo- and/or radiation therapy and tumor curability. The efficiency of cancer therapy was determined by the condition of remaining cancer tissues after treatments.

Case	Age (years)	Site	No. of chemotherapy	Radiation (cGy)	Response
1	77	Larynx	ND[a]	7020	CR
2	70	Hypopharynx	3	7020	CR
3	51	Larynx	ND	6600	CR
4	64	Maxilla	1	7000	PR
5	61	Hypopharynx	3	7000	CR
6	25	Middle ear	3	7000	NR
7	55	Nasal cavity	1	5440	NR
8	63	Retromolar trigone	3	5800	NR
9	51	Parotid	3	7020	NR

[a] ND, not done; CR, complete response; PR, partial response; NR, no response.

Fig. 2 Induction of the Prx II protein after irradiation. 11A cells were irradiated with 3 Gy, harvested at the indicated time points, and time course expression of Prx II was detected by Western blotting with a Prx II polyclonal antibody. Equal loading of the protein was confirmed by Ponceau S staining.
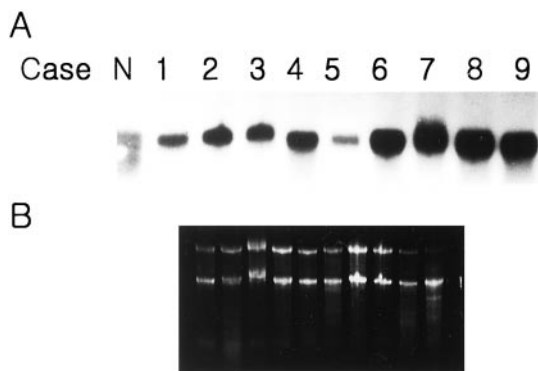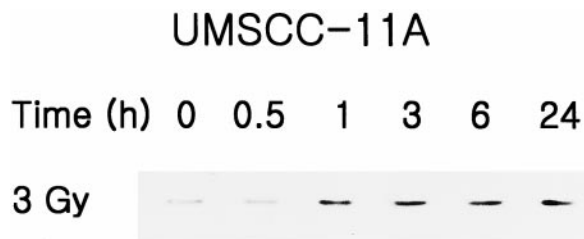

Fig. 1 Prx II expression in combined chemotherapy/radiation- or radiation therapy-resistant head-and-neck tumor tissues. Expression of Prx II in tumor tissues from the patients with head and neck cancers was detected using Northern blot procedure as described in "Materials and Methods" (*A*). Total RNA (10 μg each) was hybridized to a radiolabeled human Prx II cDNA probe. Equal loading was demonstrated with ethidium bromide staining (*B*). The profiles of the patients are listed in Table 1. *Lane N*, normal tissue from case 1; *Lanes 2–5*, radiotherapy-sensitive tumors; *Lanes 6–9*, radiotherapy-resistant tumors.

II was regulated by protein thiol-thiol exchange via the thioredoxin system, but not by GSH (15).

Delays in G_2 induced by basic fibroblast growth factor in HeLa cells have been proposed to be related to radiation resistance (16), and in Burkitt's lymphoma cells, ceramide signaling has been implicated in the radiation-resistance mechanism (17). Although the mechanisms of chemo- and/or radiation resistance in cancer cells is not well understood, we propose that inhibition of the stress-activated gene induction could be promising in efforts to overcome chemo- and/or radiation resistance. In the present study, therefore, we examined a possible role of Prx II in cellular resistance to radiation treatments and investigated a Prx II antisense as a possible radiation sensitizer in head-and-neck cancer cells.

MATERIALS AND METHODS

Cell Culture. Head and neck cancer cells (UMSCC-11A) were generously provided by R. Lotan (University of Texas M.D. Anderson Cancer Center, Houston, TX). 11A cells were cultured in DMEM/F12 (Life Technologies, Grand Island, NY) medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies).

Tissue Preparations and Northern Blot Analysis. Tumor tissues were obtained from nine patients with head and neck cancer undergoing surgical resections, rinsed with ice-cold saline solution, quickly frozen in liquid nitrogen, and stored at −70°C until needed for RNA isolation. The frozen tissues were minced with liquid nitrogen, and RNA was isolated using the phenol-chloroform method with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Ten μg of total RNA per lane were fractionated on a 1% agarose-formaldehyde gel and transferred to a nylon membrane (NEN, Boston, MA). The blot was probed with a 400-bp ^{32}P-labeled *Cla*I-*Xba*I fragment of human Prx II. Hybridization was carried out as described previously (18).

Immunoblotting. After treatment, cells were harvested and solubilized with RIPA buffer [150 mM NaCl, 1.0% NP40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris (pH 8.0)] containing protease inhibitors (1 mM sodium orthovanadate, 30 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 30 mM sodium pyrophosphate). Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). After separated on SDS-polyacrylamide gel, the proteins were transferred to nitrocellulose membranes, and the Prx II protein was detected using the anti-Prx II polyclonal antibody (7).

Construction of Sense and Antisense Prx II Expression Vector and Stable Cell Lines. A *Nde*I-*Bam*HI fragment from pETprxII (7) containing the entire coding region for Prx II was subcloned into pcDNA3 (Invitrogen, the Netherlands) to generate pPrxII/S and pPrxII/AS. pPrxII/AS contained an inverted *Nde*I-*Bam*HI fragment of the *Prx II* gene. Prx II-overexpressing cell lines were constructed in 11A cells as described previously (19). Three colonies were isolated and cultured for the use of clonogenic assay and Western blotting.

Clonogenic Assay. Exponentially growing cells were counted, diluted, and seeded in triplicate at 300 cells per culture dish (100-mm dish). Cells were incubated for 24 h in a humidified CO_2 incubator at 37°C, irradiated to γ-rays with a ^{137}Cs γ-ray source (Atomic Energy of Canada, Ltd., Ontario, Canada) at a dose rate of 3.81 Gy/min. Colonies were allowed to grow for 14 days and stained with 1% methylene blue in methanol.
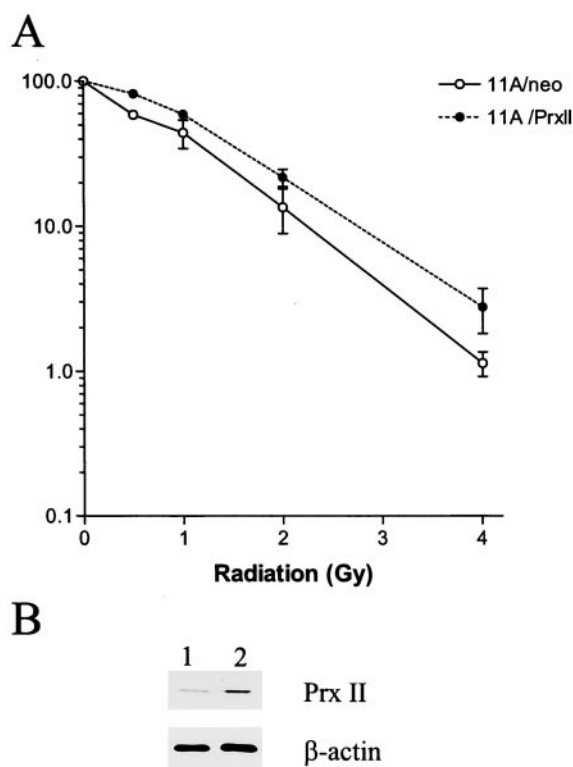
Fig. 3 Cell survival after irradiation in Prx II-overexpressing 11A cells. *A*, clonogenic survival of stable transfectant cell lines treated with radiation. Three stable transfectant cells were pooled and irradiated with 0.5, 1, 2, and 4 Gy, and cell survival was determined using a clonogenic assay as described in "Materials and Methods." Results shown are the means ± SE (*bars*) of three independent experiments. Statistical significance of the difference between 11A/neo and 11A/Prx II cells: 0.5 Gy, $P < 0.05$; 1 Gy, $P < 0.05$. *B*, Prx II expression in Prx II-overexpressing cells. Protein lysates were resolved by SDS-PAGE, and Prx II expression was analyzed by Western blotting. *Lane 1*, 11A/neo; *Lane 2*, 11A/Prx II. β-*actin* was run as a control.

Colonies larger than 200 μm in diameter were counted with a colony counter (Imaging Products, Chantilly, VA).

Radiation Sensitization of Cells. Exponentially growing cells (2×10^5) were transferred to 60-mm culture dishes and cultured for 24 h. After being washed twice with serum-free medium, the cells were incubated with a mixture containing 15 μg of cationic liposome (Life Technologies) and various doses of the anti-sense Prx II expression vector (pPrxII/AS) or anti-sense/sense oligomers (AS1, 5′-CGCGCGTTACCGGAGGC-CAT-3′; AS2, 5′-ACGCCGTAATCCTCAGACAA-3′; S1, 5′-ATGGCCTCCGGTAACGCGCG-3′) in 1 ml of serum-free medium for 4–6 h. The cells were washed with serum-free medium, added to culture medium, and then cultured for 16 h. Transfection was repeated one more time. At 16 h after transfection, the cells were irradiated to γ-rays with a ^{137}Cs γ-ray source. After 14 days of incubation at 37°C, colonies larger than 200 μm in diameter were counted with a colony counter.

Determination of Apoptotic Cell Death. For DAPI staining, 11A cells were transfected with AS1 twice and irradiated with 3 Gy. After 48 h, the cells were seeded into 100-mm tissue culture dishes. After the scheduled experiments, cells were harvested and fixed with 3.7% formaldehyde on glass slides, which were precoated with 1 mg/ml poly-L-lysine (Sigma), and stained with 1 μg/ml DAPI in PBS for 30 min. After slides were washed with PBS and mounted, the apoptotic cells were counted under a fluorescence microscope (Axioplan2; Zeiss).

For 7-AAD staining, 7-AAD (Sigma) was diluted in PBS at a concentration of 1 mg/ml as a stock solution. 11A cells were treated with either oligomers or radiation and mixed with 7-AAD stock solution to give a final 7-AAD concentration of 10 μg/ml. The cells were incubated for 20 min at 4°C in the dark and analyzed using flow cytometry (Cellquest software; Becton Dickinson). Analysis was performed as described by Schmid *et al.* (20) to select cells undergoing apoptotic death.

Statistical Analysis. Every assay was performed in triplicate and repeated at least three times. Statistical analysis was performed using the Mann-Whitney *U* test. The statistical significance of the difference between control and treatment groups was evaluated using one-way ANOVA. The criterion for statistical significance was taken as $P < 0.05$. Means, SE, and *P*s were calculated using GraphPad PRISM, version 2.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Increased Expression of Prx II in Head-and-Neck Cancer Cells after Radiation Treatment. Because Prx II was induced under oxidative stress and its overexpression had been shown to protect cells from oxidative stress (13, 14), we first investigated whether Prx II expression was related to the chemo- and/or radiation resistance in head-and-neck tumor tissues. To examine expression of Prx II and to investigate the role of Prx II in chemo- and radiation resistance, we obtained tumor tissues from two groups of patients: a chemotherapy-/radiotherapy-sensitive group and a chemotherapy-/radiation-resistant patients. For head-and-neck cancerous tumors, chemotherapeutic treatments such as cisplatin and 5-FU are commonly used prior to radiotherapy. Thus, patients were received 100 mg/m^2 cisplatin along with an injection of buffered-saline solution containing mannitol and then received 5-FU (1000 mg/m^2), followed by γ-radiation of the surviving tumors after chemotherapy. The profiles of the patients are listed in Table 1. Fig. 1 shows the strong expression of the Prx II mRNA in tumor tissues from all four patients whose tumors did not respond to combined chemo-/radiotherapy, whereas expression was weak or not induced in five of five patients whose tumors completely or partially regressed after radiation or combined chemotherapy/radiation treatments. These results suggest that increased expression of Prx II in head and neck tumors might protect the cells from radiation treatments, thereby contributing to cellular resistance to radiation treatments in cancers.

Radioprotective Effect of Prx II. To examine whether expression of Prx II was increased after irradiation, we irradiated 11A cells with 3 Gy of γ-radiation. Prx II expression in 11A cells gradually increased until 24 h after 3 Gy irradiation (Fig. 2).

Because overexpression of Prx II has been shown to protect ECV304 cells from oxidative stress induced by treatment with *t*-butylperoxide or methyl mercury (15), we transfected 11A
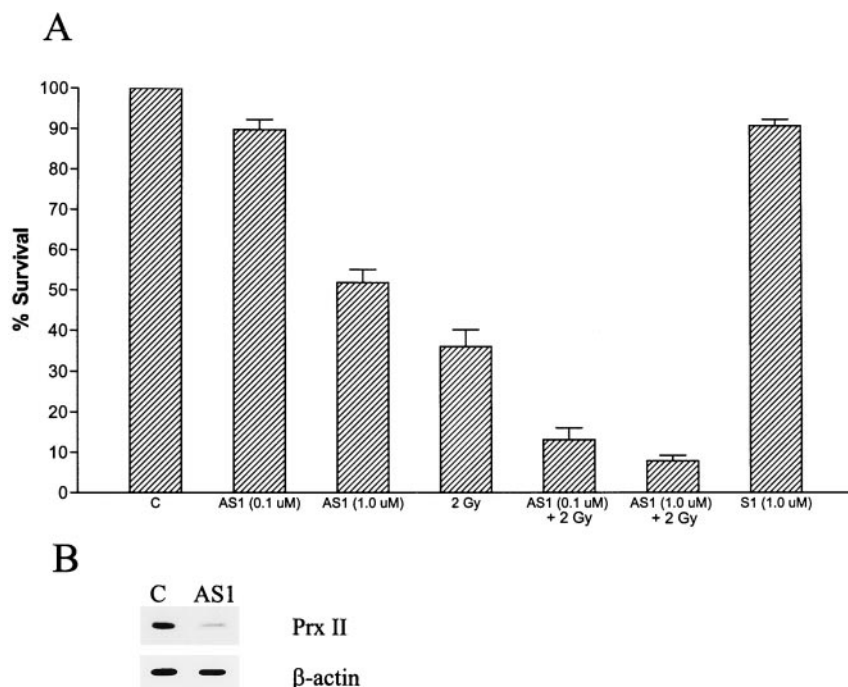
A

B

Fig. 4 Cell survival in Prx II antisense oligomer-treated 11A cells. *A*, 11A cells were transfected with AS1 and S1 twice. The cells were transfected with two different concentrations of AS1, 0.1 or 1.0 μM, before irradiation. After 2 Gy of irradiation, cell survival was measured by a clonogenic assay. *Column C*, cells transfected with no DNA; *columns AS1 (0.1 μM)* and *AS1 (1.0 μM)*, cells transfected with AS1; *column 2 Gy*, cells irradiated with 2 Gy; *columns AS1 (0.1 μM) + 2 Gy* and *AS1 (1.0 μM) + 2 Gy*, cells transfected with AS1 and then irradiated; *column S1*, cells transfected with S1. Results shown are the means ± SE (*bars*) of three independent experiments. Statistical significance of the difference between control and AS1 oligomer-transfected cells: AS1 (0.1 μM) + 2 Gy, $P < 0.001$; AS1 (1.0 μM) + 2 Gy, $P < 0.001$. *B*, inhibition of Prx II expression in Prx II antisense oligomer (AS1)-transfected 11A cells. Expression of Prx II was detected by Western blotting after transfection with AS1. *Lane C*, cells transfected with no DNA; *Lane AS1*, cells transfected with AS1. β-*actin* was run as a control.

cells with the pcDNA vector or CMV promoter carrying the open-reading frame of the Prx II cDNA to examine whether enhanced expression of Prx II protected the cells from radiation. Three colonies were pooled and referred to as 11A/PrxII. Increased expression of Prx II in the stable transfectants enhanced cell survival by ~50% after a 2-Gy dose of radiation (Fig. 3*A*). The expression profile of Prx II in the pooled transfectants was detected by Western blot analysis (Fig. 3*B*). From these data, we suggest that the induced Prx II protein could protect the cells from radiation-induced cellular damage.

Prx II Antisense as a Radiosensitizer. Because introduction of a Prx II antisense into 11A cells prevented induction of Prx II (Fig. 4*B*), antisense oligomers of Prx II were transfected into 11A cells as described in "Materials and Methods," and we examined whether down-regulation of Prx II sensitized the cells to radiation *in vitro*. Fig. 4*A* showed that with increasing dosages of a Prx II antisense (AS1) in 11A cells, the more cell death was observed. Treatment of a second antisense (AS2) targeting the other open-reading frame sequence of the Prx II cDNA showed a result similar to that for AS1 treatment (data not shown). Treatment of 11A cells with a Prx II sense oligomer (S1) did not alter cell viability compared with the control cells. These results demonstrated that a Prx II antisense caused cell death, which was dependent on the concentration of a Prx II antisense used.

Although ~37% of 11A cells survived after 2 Gy of irradiation, and ~90% survived after treatment with 0.1 μM Prx II antisense (AS1), cell survival decreased to ~13% after 2 Gy of irradiation in the presence of 0.1 μM Prx II antisense and to ~8% with irradiation in the presence of 1.0 μM Prx II antisense (Fig. 4*A*). At 1.0 μM, Prx II antisense itself caused cell death in ~48% of 11A cells.

To confirm whether down-regulation of Prx II enhanced

cell death after irradiation, 11A cells were transiently transfected with the antisense Prx II expression vector containing the full-length antisense Prx II cDNA at three different concentrations. When the cells were irradiated with 2 Gy of γ-radiation in the presence of 0.1 μg/ml pcDNA3, it increased cell death by ~53% compared with no γ-radiation. However, 0.1 μg/ml pPrxII/AS with 2 Gy of γ-radiation increased cell death by ~89% (Fig. 5). Thus, transfected cells with a Prx II antisense showed a synergistic increase of cell death after radiation compared with parent cells transfected with pcDNA3. It is apparent from these data that expression of Prx II can protect cells from radiation-induced cell death and, therefore, that radiation sensitivity can be increased by the inhibition of Prx II expression.

Prx II Antisense-induced Apoptosis. To demonstrate that the presence of a Prx II antisense could stimulate apoptosis and enhance cellular damage after radiation, apoptotic cell death was examined by flow cytometry using uptake of the dye 7-AAD after treatment of 11A cells with a Prx II antisense. Fig. 6*A* shows that Prx II antisense alone could induce apoptosis. Furthermore, increased apoptotic cell death was observed in the irradiated 11A cells pretreated with a Prx II antisense. We also examined apoptotic cell death by DAPI staining. The irradiated cells pretreated with Prx II antisense demonstrated chromosome condensation, which was indicative of apoptosis (Fig. 6*B*).

DISCUSSION

Although DNA-damaging agents such as γ-radiation and chemotherapeutic drugs have been widely used for the treatment of numerous cancers, cells increasingly become resistant to consecutive administration of chemotherapeutic drugs and radi-
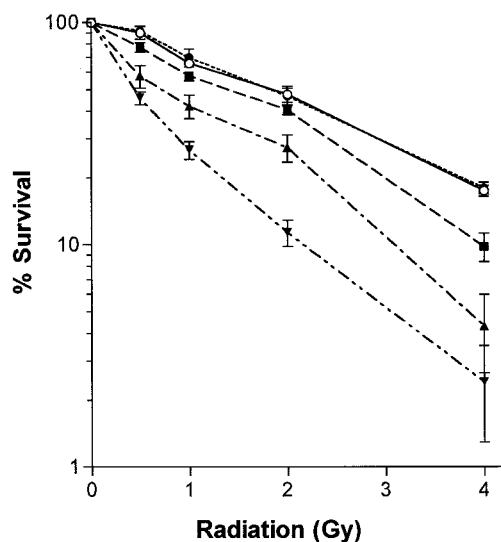
Fig. 5 Radiation sensitization of 11A cells after transfection with an antisense Prx II expression plasmid (pPrxII/AS). 11A cells were transfected with 0.025 (■), 0.05 (▲), and 0.1 μg/ml (▼) pPrxII/AS, as described in "Materials and Methods." The cells were then irradiated with 0.5, 1, 2, and 4 Gy. Cell survival was measured by a clonogenic assay. As a control, the cells were transfected with 0.05 μg/ml of sense Prx II expression plasmid (pPrxII/S; ●). Results shown are the means ± SE (*bars*) of three independent experiments. Statistical significance of the difference between control (pcDNA3; ○) and pPrxII/AS (0.1 μg/ml; ▼)-transfected cells: 0.5 Gy, $P < 0.004$; 1, 2, and 4 Gy, $P < 0.001$.

Fig. 6 Apoptosis induced by Prx II antisense. *A*, 11A cells were treated with S1, AS1, or 3 Gy of radiation, then stained with 7-AAD. *S1*, cells transfected with 1 μM S1; *AS1*, cells transfected with 1 μM AS1; *3 Gy*, cells irradiated with 3 Gy; *AS1+3 Gy*, cells irradiated with 3 Gy after transfection with 1 μM AS1; *a*, apoptotic cells; *d*, dead cells. *FCS-H*, forward scatter-height. *B*, 11A cells were transfected with AS1, irradiated with 3 Gy, and then stained with DAPI.

ation. Therefore, studies involving strategies to overcome this resistance to chemotherapy and radiation or to increase cellular sensitivity to chemo- and radiation therapy contribute a critical step for better cancer treatments. In this study, we showed enhanced expression of Prx II in head-and-neck cancer cells that are chemotherapy- and/or radiation-resistant and showed the possible use of Prx II antisense as a radiosensitizer. Induction of the ROS-activated proteins could protect cells from oxidative cytolysis and reduce cellular damage by regulating cellular redox status (21–24). Baker *et al.* (25) observed that the redox protein thioredoxin was overexpressed in human primary lung and colon tumors and that it prevented apoptosis through the antioxidant mechanism. We also observed strong expression of Prx II in tumor tissues isolated from patients with chemotherapy- and/or radiation therapy-resistant tumors, whereas expression of Prx II was weak in the chemotherapy-/radiation-sensitive tumor tissues, which regressed after radiotherapy (Fig. 1). Similar to increased expression of Prx II in irradiated 11A cells, the expression of Prx II in 11A cells was also enhanced by chemotherapeutic drugs such as cisplatin (5 μg/ml) and 5-FU (2.5 μg/ml; data not shown). It is therefore likely that intracellular ROS induced by cisplatin, 5-FU, and radiation increase expression of Prx II in cancer cells, thereby endowing the cancer cells with resistance. It should be noted here that overexpression of Prx II has been observed to give more resistance to the chemotherapeutic drug CT-2584 in ECV304 cells (12), and we also observed strong expression of Prx II in cisplatin-resistant stom-
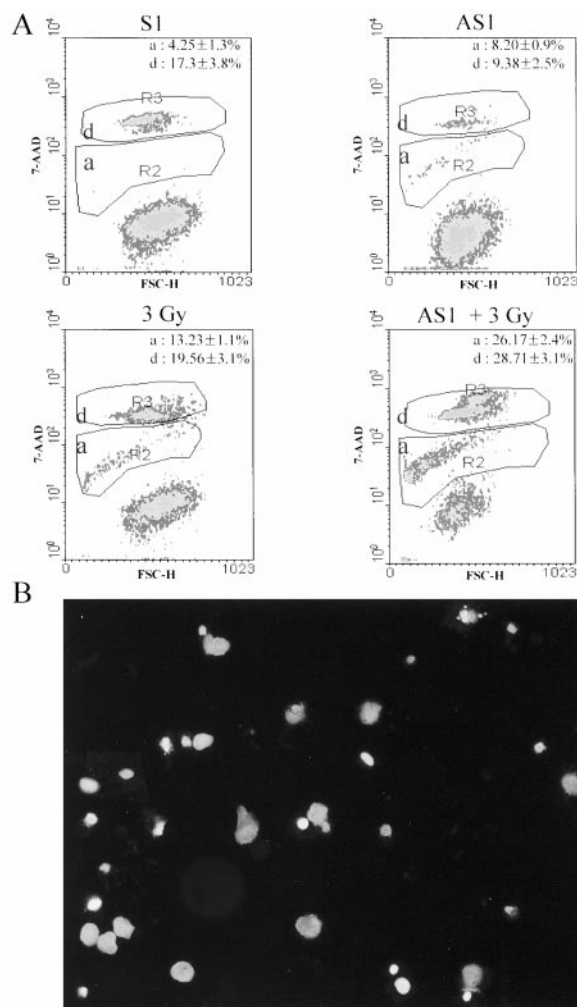
ach cells compared with parent cells.[6] From the above considerations, we propose that Prx II functions not only as an antioxidant protein but also as a stress-activated protein.

Prx II protein has been proposed to play a role as a thiol-specific antioxidative protein (15) that reacts with thiol-containing proteins such as thioredoxin. Because cellular thiol-specific proteins are considered to regulate the activity of other cellular proteins with thiols at regulatory domains (21, 24), inhibition of Prx II activity could possibly affect signaling pathway for cell growth and survival. Disruption of cellular redox status has been shown to activate the caspase 3 cascade, resulting in apoptosis (22). Zhang *et al.* (8) showed that over-

[6] S-H. Park, Y. M. Chung, and D. Y. Young, unpublished data.

expression of Prx II inhibited apoptosis in Molt-4 leukemia cells and functioned similarly to Bcl-2. One difference in function between Prx II and Bcl-2 is that Prx II can prevent accumulation of H_2O_2 in cells, thereby protecting cells from H_2O_2-induced cell death. These observations could explain how treatment of 11A cells with a Prx II antisense induces apoptotic cell death. Inhibition of Prx II expression did not influence the intracellular concentration of GSH, an indicator of cellular redox status, in irradiated 11A cells treated with a Prx II antisense (data not shown). This observation was consistent with a previous result that activity of Prx II was not regulated by intracellular concentrations of GSH (13). Therefore, blocking the expression of Prx II might disrupt total cellular redox homeostasis but not the GSH concentration, resulting in apoptosis (24). It should again be emphasized that because overcoming radiation resistance is critical for better cancer treatment, inactivation of the stress-activated protein Prx II may be a promising approach to increased radiation sensitivity in head-and-neck cancer cells.

ACKNOWLEDGMENTS

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