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Original Research Communication

Induction of Peroxiredoxins in Transplanted Livers and Demonstration of Their *In Vitro* Cytoprotection Activity

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ABSTRACT

Peroxiredoxin (Prx)-I and -II belong to a new class of antioxidants. Here, we report that they are induced by ischemia/reperfusion (I/R) in transplanted livers. Hypothesizing that Prxs are induced to protect liver from oxidative damage, we transduced these human genes into murine NIH-3T3 cells. The overexpressed Prxs made the cells more resistant to *t*-butylhydroperoxide-induced apoptosis. These results indicate that Prx-I and Prx-II are induced by the transplantation process and can protect cells against oxidant damage in tissue culture. Thus, proper genetic manipulations of Prxs may be useful in increasing the success rate of organ transplantation. Antiox. Redox Signal. 2, 347–354.

INTRODUCTION

ORGAN TRANSPLANTATION from donor to recipient involves ischemia and reperfusion (I/R), cutting off and later reconnection of the blood supply. I/R of liver causes enhanced mitochondrial production of reactive oxygen species (ROS) that leads to lipid peroxidation as well as damage to proteins and nucleic acids. Among many diverse effects, cytokine release, neutrophil adhesion, sinusoid endothelial cell death, and hepatocyte injury are the most likely means to manifest these events (Serizawa *et al.*, 1996; Bzeizi *et al.*, 1997).

Cells employ antioxidants not only to protect themselves from oxidant toxicity but also to manipulate signal transduction for controlling gene expression (Mihm *et al.*, 1995; Halliwell, 1996; Sen and Packer, 1996). Peroxiredoxins (Prxs) are a newly discovered family of antioxidants that are well conserved evolutionarily (Rhee *et al.*, 1994; Shau *et al.*, 1997; Kang *et al.*, 1998; Schreoder *et al.*, 1998; Butterfield *et al.*, 1999). Over several hundred Prxs have been discovered in species ranging from bacteria to human beings. They are divided into two subfamilies, depending on their requirement for antioxidation activity; one that has two conserved cysteines (Prx-I–Prx-IV) and the other that has a single conserved cysteine (Prx-V and Prx-VI). Among the human Prxs, Prx-I to Prx-IV have been the most studied so far.

We originally cloned human Prx-I and Prx-II because of their immunoregulatory function. Although both proteins have comparable capacity to scavenge ROS, we have documented

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that only Prx-I is capable of augmenting natural killer activity (Sauri *et al.*, 1996). There are several stress proteins from different species that share homology with Prx-I and Prx-II and are often induced by oxidant and inflammatory stress (Sato *et al.*, 1993; Immenschuh *et al.*, 1997; Kim *et al.*, 1997; Kang *et al.*, 1998; Schreoder *et al.*, 1998). We, as well as others, have reported that Prx-II functions as feedback control to protect cells from ROS toxicity (Kang *et al.*, 1998; Sarafian *et al.*, 1997; Shau *et al.*, 1997, 1998; Zhang *et al.*, 1997). Prx-II also blocks the NF-κB and AP-1 signal transduction pathways that are activated by ROS and by tumor necrosis factor-α (TFN-α).

Normal cellular antioxidant defense involves a delicate balance of several enzyme systems that are increasingly recognized as critical regulators of cell function and viability (Halliwell, 1996; Sen and Packer, 1996; Nakamura *et al.*, 1997). Perhaps part of the problem with patients subjected to liver transplants is the low levels of normal antioxidant mechanisms present in the liver. Therapeutic interventions directed at altering the influence of oxidative stress in transplanted livers may have significant advantages compared to other organs. In the murine models, oxidative stress or partial hepatectomy induces the expression of Prx-I in liver cells (Iwahara *et al.*, 1995). Because Prx-II is induced by hydrogen peroxide in human endothelial ECV304 cells (Kim *et al.*, 1997) and oxidative stress is a major consequence of I/R, we decided to investigate the expression of Prx-I and Prx-II in transplanted liver. We report here that I/R induces Prx-I and Prx-II in transplanted liver. Furthermore, overexpression of Prx-I and Prx-II protects fibroblasts from organic oxidant-induced apoptosis.

MATERIALS AND METHODS

Wedge liver biopsy specimens were obtained immediately prior to reperfusion, and at timed intervals thereafter. All specimens were divided and subsequently snap-frozen in liquid nitrogen. Protein extracts from liver specimens and cell lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After blotting the proteins onto nitrocellulose membranes, we probed the membranes with anti-Prx primary monoclonal antibodies (mAbs) (Sarafian *et al.*, 1999). Details of immunoblotting have been described previously (Shau *et al.*, 1997). Protein bands reactive with specific antibodies were detected with either enhanced chemiluminescence (ECL) or nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-iodolyphosphate-p-toluidine (BCIP) substrate.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on total liver RNAs extracted by RNeasy (Qiagen, Valencia, CA) with the SuperScript II reverse transcriptase and Platinum Taq (Life Technologies, Gaithersburg, MD) according to the supplier's protocols with minor modifications. The primer pairs are 5'CTTGTTCTTGCCTGGTGTCGG-3'/5'-GGTGCGCTTCGGGTCTGATA-3' for Prx-I with an expected 397-bp product, and 5'-GTCTGAGGATTACGGCGTGCTG-3'/5'-ATCGTGTCACTGCCAGGCTTCC-3' for Prx-II with an expected 217-bp product. Ambion (Austin, TX) 18S primer pair and 18S competimers with the expected 488-bp product were used at the ratios of 2:3 and 3:7 for quantifying Prx-I and Prx-II messages, respectively. The PCR products were resolved on 2% agarose gels, stained with Sybr-Green (Molecular Probe, Eugene, OR), and detected with 300-nm UV. Images were captured with a Kodak DC 120 digital camera. Gel band densities were determined using Kodak 1D gel image software V.2.0.

The NIH-3T3 subline MT was infected with MSCV-PrxI-ires-GFP and MSCV-PrxII-ires-GFP. We obtained Prx-I and Prx-II DNAs covering their respective open reading frames from previously isolated p21 and p5 clones (Shau *et al.*, 1994). These DNAs were inserted into the *Eco* RI cloning site upstream of the internal ribosome entry site (ires) in the MSCV-ires-GFP retroviral vector (Fig. 1), which is a modified version of a previously described vector (Hawley *et al.*, 1994). We produced high-efficiency recombinant retrovirus for the Prx genes by co-transfecting 293T cells with the respective retroviral vectors and the packaging ψ-ecotropic helper plasmid. Retroviruses were collected every 5 hr for 20 hr, pooled, and used for infecting the 3T3 cells.

To test for resistance to oxidant toxicity, we treated 2 to 10×10^3 cells with the organic oxidant *t*-butylhydroperoxide (TBHP) in microwells for 24 hr. Cell viability was measured by acid phosphatase activity (Connolly *et al.*,

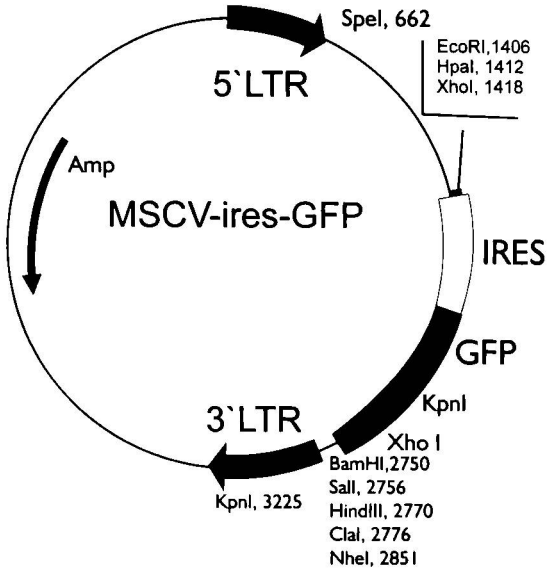

FIG. 1. Core structure of MSCV-ires-GFP. Human Prx-I or Prx-II gene was inserted at the EcoRI site upstream of ires. Positions of the restriction enzyme sites are indicated by numbers following the names of the enzymes. GFP, green fluorescent protein; ires, internal ribosomal entry site.

1986). Cell death is indicated by the decrease of acid phosphatase activity relative to cells treated with control medium alone in duplicate assay and is expressed as percent viability ± SEM.

Apoptotic death was measured by staining the cells with 7-amino-actinomycin D (7AAD) (Schmid *et al.*, 1994). After treating the cells with TBHP, we stained them with 2 μg/ml of 7AAD in cold Dulbecco's phosphate buffer saline (PBS) for 20 min in the dark. After being washed once with PBS, the cells were fixed with 1% paraformaldehyde in cold PBS containing 4 μg/ml of actinomycin D. Stained cells were analyzed by flow cytometry.

RESULTS

Liver damage has been shown to induce expression of Prx-I in animal models (Iwahara *et al.*, 1995). Therefore, we sought to explore the effect of I/R on Prx expression in transplanted human livers. Serial specimens from two successful liver transplantation cases were used for study. We extracted proteins from the biopsies and separated them by SDS-PAGE. Immunoblot results of one of the two cases with mAbs specific for Prx-I and Prx-II are shown in Fig. 2. It is demonstrated that the levels of both Prx-I and Prx-II were increased after I/R, and that the increase became greater as time progressed post transplantation of the liver. RT-PCR results indicated that the messages of both Prx-I and Prx-II were significantly increased relative to that of 18S ribosomal RNA (Fig. 3). However, the messages of both genes reached plateau at 30 min post reperfusion, while their protein levels continued to rise. Similar results were observed in the second case of liver transplantation. In both cases, I/R appeared to induce the expression of Prx messages and proteins.

It is well established that I/R causes oxidative damage in liver. Because both Prx-I and Prx-II are antioxidants, we hypothesized that these proteins were induced to protect liver cells. To test for the Prx cytoprotective function, we introduced these two human genes into mouse 3T3 fibroblast cells with MSCV-ires retroviral vectors. The mouse MT subline was selected because it is easy to be manipulated and has low background for detection with our mAbs specific for human Prx proteins. After infection with retroviral vectors carrying Prx-I or Prx-II genes, the cells were analyzed at specific days post infection by flow cytometry to detect the green green fluorescence protein (GFP) signal (Fig. 4). The results show that GFP$^+$ cells increased from 88% on day 3 for both Prxs to 99% and 98% on day 20 for Prx-I and Prx-II transduced populations, respectively. Because Prx genes preceded ires that was upstream of

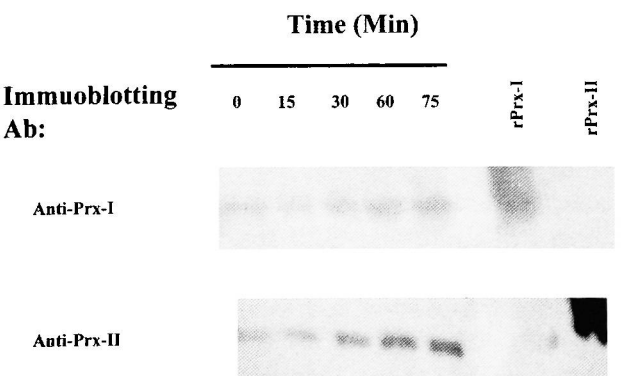

FIG. 2. I/R induction of Prx proteins in transplanted human liver. Liver specimens were obtained at indicated time points before and after reperfusion, extracted for proteins, then immunoblotted with mAbs specific for Prx-I and Prx-I, respectively. Recombinant (r) proteins were included as positive controls.

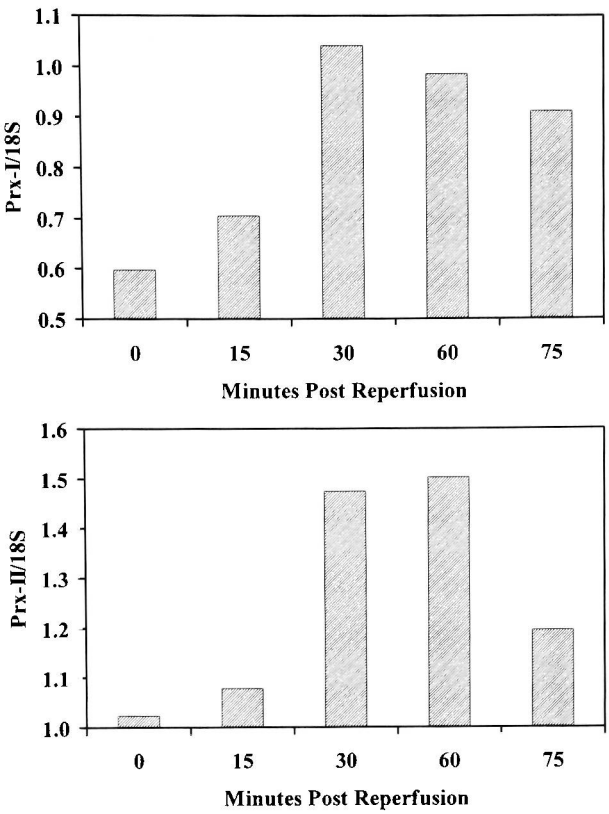

FIG. 3. I/R induction of Prx messages in transplanted human liver. RT-PCR was performed with total RNAs extracted from separate portions of the same liver specimens used for immunoblotting in Fig. 2. The ratios indicate the relative amount of Prx products to that of the 18S ribosomal RNA. Top panel, Prx-I/18S RNA ratios; bottom panel, Prx-II/18S RNA ratios.

GFP, all green cells are presumably overexpressing the transduced Prx proteins. To confirm the overexpression of Prxs, we separate the protein extracts by SDS-PAGE and then immunoblotted the membranes with Prx-specific mAbs. Results in Fig. 5 indicate that indeed each of the GFP^+ cells overexpressed its respective upstream Prx protein. This experiment was repeated twice with similar results. These data demonstrate the high efficiency of the MSCV-ires retroviral vectors for introducing Prxs linked to the GFP marker. These transduced sublines now became useful tools to test for Prx cytoprotective function against oxidant toxicity.

We treated the parental MT and its Prx overexpressing sublines with the organic oxidant TBHP then assay for their viability. As shown by the data in Fig. 6, more Prxs-overexpressing cells survived TBHP toxicity than the MT parental cells at low concentrations of TBHP. Three other experiments of TBHP treatment showed similar resistance of Prx-overexpressing cells. Low concentrations of oxidants have been shown to induce apoptotic cell death (Gardner *et al.*, 1997). One hallmark of apoptosis is that cell membrane becomes permeable to the DNA dye 7AAD much earlier (within hours) than disintegration of the whole cell structure (up to days). Flow cytometry analysis with 7AAD staining showed that TBHP was more toxic to the MT control cells than the Prx-overexpressing cells. Results in Table 1 demonstrate the concentration dependency of TBHP toxicity and the protection of cells by overexpressed Prx proteins. Similar results were repeated with up to 5 hr of exposure of 40 μM TBHP and enumeration with fluorescent microscope examination.

DISCUSSION

In this study we found that I/R induces overexpression of Prx-I and Prx-II in transplanted livers. These results are consistent with previous reports of murine models where partial hepatectomy induced the expression of Prx-I (Iwahara *et al.*, 1995). Because human Prx-I was also cloned by others as a proliferation associate gene (Prospaeri *et al.*, 1993), one would expect the murine Prx-I to be highly augmented during the liver regeneration caused by partial hepatectomy. However, Prx-II appears to have similar response to I/R insult (Figs. 2 and 3). Thus, we conclude that I/R and other growth stimulatory insult can generally increase the expression of Prx-I and Prx-II in liver.

In our tissue culture model with 3T3 fibroblasts, both Prx-I and Prx-II gave growth advantage over parental MT cells. After co-transduction of Prx and GFP genes, the transduced cells started to take over the cell culture and their proportion grew to nearly 100% within 1 month (Fig. 4). Furthermore, the transduced cells yielded higher numbers of cells than the parental cells when seeded with same numbers of cells under identical conditions (data not shown). Thus, both Prx-I and Prx-II can provide cell growth advantage over the control parental line.

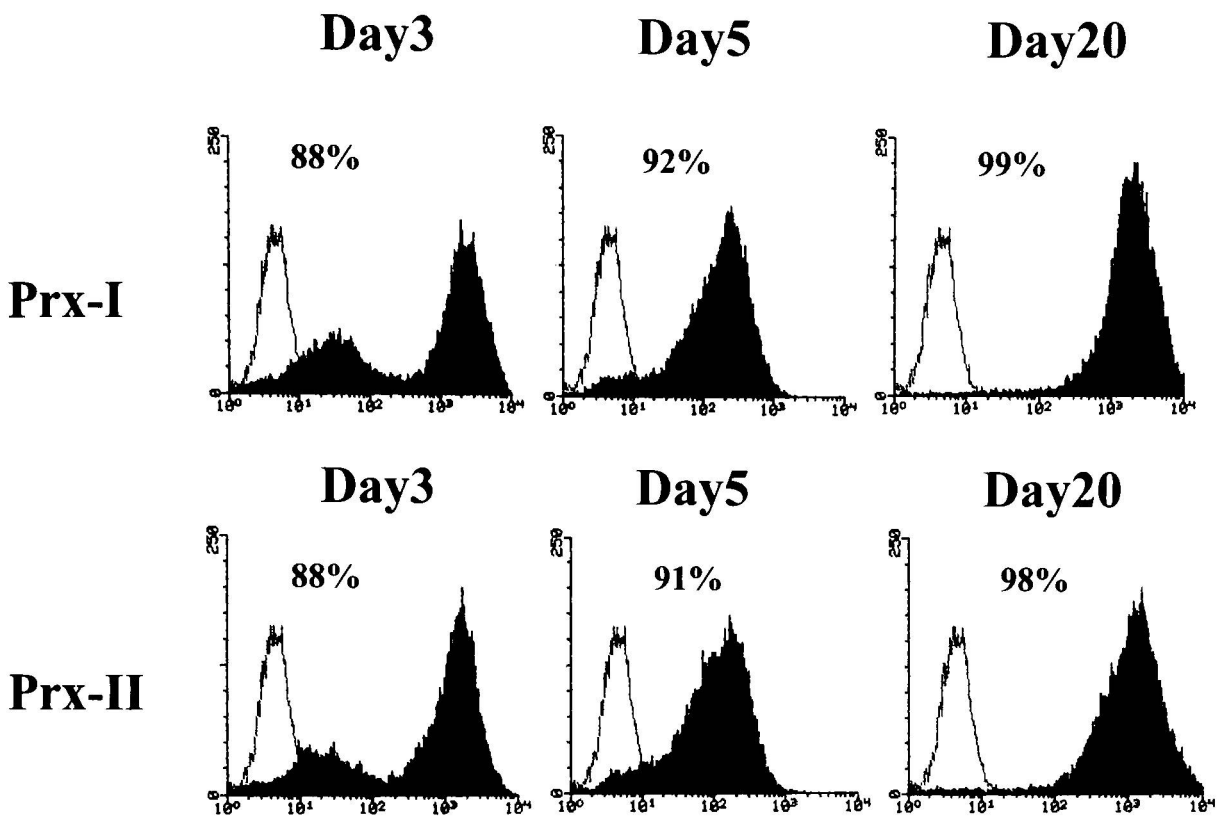

FIG. 4. Flow cytometry analysis of GFP in Prx-I- and Prx-II-transduced 3T3 MT cells. The expression of GFP was analyzed on the indicated days post infection with MSCV-ires-GFP retroviral vectors carrying human PRX-I or Prx-II gene upstream of ires. *x*-axis, fluorescence intensity; *y* axis, cell number. The number in each panel indicates percentage of GFP-positive cells.

Another significant finding here was the demonstration that not only Prx-II, but also Prx-I, can protect cells from organic oxidant toxicity. Western blotting and GFP intensity suggest that Prx-I$^+$ cells express greater level of the transduced genes than Prx-II$^+$ cells. However, the Prx-II$^+$ cells are consistently more resistant to TBHP toxicity (Fig. 6). Previously, we reported that Prx-II protects cells from oxidant toxicity (Shau *et al.*, 1997). Zhang *et al.* (1997) further documented that Prx-II inhibits oxidant-induced apoptosis via blockage of caspase-3 activation. Their data suggested that Prx-II blocks caspase-3 activation by preventing the release of essential cytochrome *c* from mitochondria. We are currently confirming these results with Prx-I and investigating how the cytosolic Prx-I and Prx-II interfere with the mitochondrial reactions.

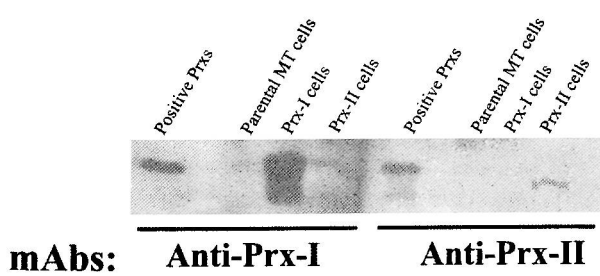

FIG. 5. Prx-I and -II overexpression in respectively transduced cells. Protein extracts from 3T3 MT cells infected with MSCV-Prx I-ires-GPF and with MSCV-Prx II-ires-GFP were immunoblotted and probed with mAbs specific for the two human proteins. Purified native Prxs (containing both Prx-I and -II) from red blood cells was included as a positive control.

Although Prxs can scavenge both organic and inorganic hydroperoxides (Rhee *et al.*, 1994; Sauri *et al.*, 1995; Netto *et al.*, 1996; Poole *et al.*, 1997; Sarafian *et al.*, 1997; Kang *et al.*, 1998), the much better known catalase is only capable of disposing of inorganic hydrogen peroxide. It is possible that during the evolution these antioxidants are separately selected for protection against their specifically sensitive ROS (Chae *et al.*, 1999). In our previous

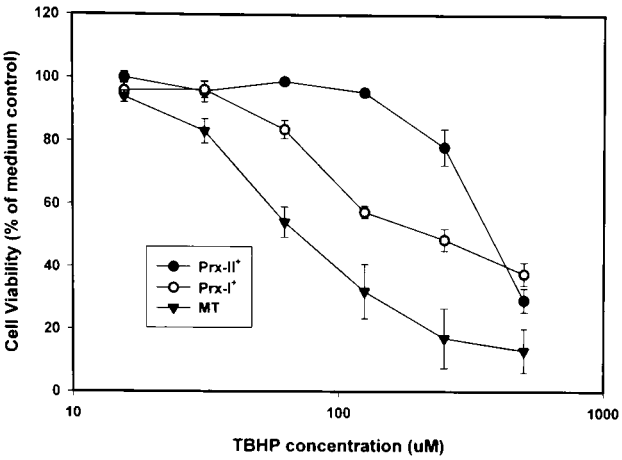

FIG. 6. Prx-I and Prx-II protection of 3T3 MT cells against oxidant toxicity. The 3T3 sublines were cultured with indicated concentrations of TBHP, then tested for acid phosphatase activity as indicator of viability. (SEM not shown when ≤2%.)

studies, Prx-II protection appeared to be more effective against the toxicity of the organic TBHP than the inorganic hydrogen peroxide in the human endothelial ECV304 cells (Kim *et al.*, 1997; Sarafian *et al.*, 1997; Shau *et al.*, 1997). In the current study, we also detected greater Prx protection against the organic than inorganic oxidants in the murine 3T3 cells (data not shown). Thus, the distinction of cytoprotection against organic versus inorganic oxidants may be a separation of labor due to natural selection.

It is important to keep in mind that the growth effect and protective ability of Prxs most likely depend on the genetic background and tissue specificity. Both Prx-I and Prx-II need thioredoxin *in vivo* and other thiol reagents *in vitro* for the reducing equivalents to scavenge hydroperoxides. Thioredoxin itself can accept electrons from NADPH to protect cells (Rhee *et al.*, 1994; Nakamura *et al.*, 1997). Certain animal strains and tissues may not have sufficiently abundant thioredoxin or NADPH for the reducing equivalents. In those cases, we are unlikely to detect growth advantage or cytoprotection with overexpression of Prxs.

TABLE 1. PROTECTION OF CELLS AGAINST TBHP-INDUCED APOPTOSIS BY PRXS[a]

TBHP	*%7AAD+*		
(μM)	*MT*	*Prx-I+*	*Prx-II+*
0	0	0	0
10	0.6	0.9	0
20	8.7	1.0	0.6
40	25	8.2	6

[a]Cells were treated with indicated concentrations of TBHP for 3 hr at 37°C, washed, and stained with 7AAD. Apoptotic cells were detected by flow cytometry and presented as percent positive after subtracting the background staining of 3%, 0%, and 1% for MT, Prx-I+, and Prx-II+ cells, respectively.

Antioxidation is not the only biological function that Prxs have. Survival of natural killer cells depends on their redox status (Furuke *et al.*, 1999). Prx-I, but not Prx-II, stimulates natural killer activity (Sauri *et al.*, 1996). The fact that Prx-I and -II have comparable antioxidation capacity (Sauri *et al.*, 1995) suggests that Prx-I modulates natural killer function via a redox independent mechanism. In contrast Prx-II, but not Prx-I, was identified as an enhancer of calcium-activated potassium channel in red blood cell membrane (Kristensen *et al.*, 1999; Moore and Shriver, 1997; Schreoder *et al.*, 1998). Such discrepancies between these two proteins are probably due to their distinct basic biological functions independent of antioxidation activity. Another example of non-antioxidant function by Prxs was reported by Wen and van Etten (1997). They generated Prx-I mutants by truncating its sequences essential for antioxidation function. They found that, like the wild-type Prx-I, those antioxidation-defective mutants were still able to bind the proto-oncogen c-*abl* product and inhibit c-Abl kinase activity as well as its ability to regulate cell growth. Therefore both Prx-I and Prx-II have multiple domains that we can selectively manipulate for regulating their biological functions.

Besides cytoprotection, Prxs can also affect the survival of transplanted livers by other mechanism. Prx-I enhances natural killer activity (Sauri *et al.*, 1996) and natural killer cells are major effectors in rejecting allogeneic transplants (Katznelson *et al.*, 1998; Trinchieri, 1989). Pravastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, has become a popular drug in prolonging the survival of transplanted organs in recent years (Katznelson *et al.*, 1998). We documented that in the rat liver transplantation model, a combination of Pravastatin and cyclosporin A is much more effective than cyclosporin A alone in preventing organ rejection (Kakkis *et al.*, 1997). Although the mechanism of Pravastatin immunosup-

pression is not clear, it is suspected that inhibition of natural killer activity is of major significance. This hypothesis is especially attractive because the decrease of natural killer activity is correlated with Pravastatin's lowering Prx-I and Prx-II expression in the same liver recipients. The original observation of Prx-I and Prx-II up-regulation was made within weeks of liver transplantation in allogeneic animals (Kakkis *et al.*, 1997) that we believe is induced by chronic immune responses. In contrast, the increase of Prxs in human livers happened within hours. We are currently using a syngeneic rat liver transplant model to define further the observed acute I/R responses of Prxs. If Prx-I and Prx-II are indeed induced in two phases, acute I/R and chronic immune responses, these two gene products offer great potential for genetic and biochemical intervention of liver function in transplantation biology. By blocking Prx-I-mediated enhancement of natural killer activity, one would reduce the risk of transplant rejection by the recipients. On the other hand, Prx antioxidation activity can be preserved or increased to protect cells from undergoing apoptosis. This strategy would afford protection of transplanted organs from I/R damage, thus preventing many of the incidents where donor organs need to be discarded due to delay by time or distance.

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ABBREVIATIONS

7AAD, 7-amino-actinomycin D; GFP, green fluorescence protein; I/R, ischemia/reperfusion; ires, internal ribosomal entry site; mAb, monoclonal antibody; PBS, phosphate-buffered saline; Prx, peroxiredoxin; r, recombinant; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBHP, *t*-butylhydroperoxide; TNF-α, tumor necrosis factor-α.

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