Title

DELIVERY OF ANTIOXIDATIVE ENZYME GENES PROTECTS AGAINST ISCHEMIA/REPERFUSION-INDUCED LIVER INJURY IN MICE

Journal

Liver Transplantation, 12(12)

Author

Wu, Jian

Publication Date

2007

Peer reviewed

tapraid5/zlt-liver/zlt-liver/zlt00107/zlt1119d07g | heckt | S=5 | 10/20/06 | 9:18 | Art: 06-320

AQ: 2

ORIGINAL ARTICLE

Delivery of Antioxidative Enzyme Genes Protects Against Ischemia/Reperfusion–Induced Liver Injury in Mice

Song-Qing He,[1] Yan-Hong Zhang,[1] Senthil K. Venugopal,[1] Christopher W. Dicus,[2] Richard V. Perez,[3] Rajen Ramsamooj,[4] Michael H. Nantz,[2] Mark A. Zern,[1] and Jian Wu[1]

[1]Transplant Research Institute, University of California, Davis Medical Center, Sacramento, CA; [2]Department of Chemistry, University of California, Davis, Davis, CA; [3]Department of Surgery, University of California, Davis Medical Center, Sacramento, CA; [4]Department of Pathology, University of California, Davis Medical Center, Sacramento, CA

Hepatic ischemia/reperfusion (I/R) injury is characterized by the generation of reactive oxygen species (ROS), such as superoxide anions and hydrogen peroxide. The aim of this study is to investigate whether antioxidative gene delivery by our polylipid nanoparticles (PLNP) is an effective approach for prevention of the injury. Polyplexes of extracellular superoxide dismutase (EC-SOD) and/or catalase genes were injected via the portal vein 1 day prior to a warm I/R procedure in mice. The effects of the gene delivery were determined 6 hours after starting reperfusion. PLNP-mediated antioxidative gene delivery led to a marked increase in human EC-SOD and catalase gene expression in the liver. Liver superoxide dismutase (SOD) and catalase activity both increased approximately 10-fold. Increased liver superoxide anion levels caused by the I/R procedure were reduced to normal levels by EC-SOD gene delivery. The overexpression of these 2 antioxidative genes significantly suppressed the I/R-induced elevation of serum alanine aminotransferase (ALT) levels, decreased liver malondialdehyde content, restored glutathione reserve, and improved liver histology. In conclusion, EC-SOD or catalase gene delivery by PLNP resulted in high levels of the transgene activity in the liver, and markedly attenuated hepatic I/R injury. The protection is directly associated with elevated antioxidative enzyme activity as the result of the gene delivery. This novel approach may become a potential therapy to improve graft function and survival after liver transplantation. ***Liver Transpl 12:000-000, 2006.***

Received June 16, 2006; Accepted August 29, 2006.

Ab

Ischemia/reperfusion (I/R)-induced liver injury is a process whereby hypoxic organ damage occurs following the return of blood flow and oxygen delivery.[1-3] Transient episodes of ischemia are encountered during liver transplantation, trauma, hypovolemic shock, and surgical liver resection, when inflow occlusion or total vascular exclusion is used to minimize blood loss. The pathogenesis of hepatic I/R injury has been studied for nearly 3 decades, and many crucial elements of the pathogenesis have been identified.[1,4] One key element of the pathologic alteration in hepatic I/R injury is the generation of reactive oxygen species (ROS) during the reperfusion, which contributes to hepatocyte and sinusoidal endothelial cell damage (1,4).

Superoxide anion (O_2^-) is one of the major ROS. The release of O_2^- in hepatic I/R injury was proven when allopurinol, a specific inhibitor of xanthine oxidase, attenuated hepatic I/R injury.[5] The importance of O_2^- is

Abbreviations: I/R, ischemia/reperfusion; ROS, reactive oxygen species; PLNP, polylipid nanoparticle; EC-SOD, extracellular superoxide dismutase; SOD, superoxide dismutase; ALT, alanine aminotransferase; DNA, deoxyribonucleic acid; MCLA, 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo [1,2-α] pyrazin-3-one); GSH, reduced form of glutathione; MDA, malondialdehyde; N.S., normal saline; AP-1, _____; NF-κB, nuclear factor-κB; TNF-α tumor necrosis factor-α

AQ: 1

Presented in part at the 9th Annual Meeting of the American Society of Gene Therapy, May 31-June 4, 2006, Baltimore, MD, and the abstract was published in *Molecular Therapy* 2006;13(Suppl):S415.
Supported by National Institutes of Health (NIH) grants (DK069939 to J.W. and AA06386 to M.A.Z.).
Address reprint requests to Jian Wu, MD, PhD, UC Davis Medical Center, Transplant Research Institute, 4635 2nd Ave. Suite 1001, Sacramento, CA 95817. Telephone: (916) 734-8044; FAX: (916) 734-8097; E-mail: jdwu@ucdavis.edu

DOI 10.1002/lt.21001
Published online in Wiley InterScience (www.interscience.wiley.com).

also indicated by the fact that a manganese superoxide dismutase (SOD) mimetic and genetically engineered manganese-SOD inhibited I/R-induced liver injury by 60 to 70%.[6] O_2^- is a central and initial species that is readily converted to other species when SOD activity is inadequate.[7] A significant amount of O_2^- is thought to be generated by infiltrative neutrophils, activated Kupffer cells, and damaged hepatocytes during the I/R process.[1,8,9] O_2^- may be released into the extracellular space when cells are injured and thus causes toxicity to other surrounding cells. Accumulated O_2^- in the extracellular space may be converted to more toxic H_2O_2, HO^-, or $ONOO^-$ in the presence of H^+, H_2O_2, and nitric oxide. These O_2^--derived ROS participate in the inflammatory process, and are thought to be key mediators for the activation of Kupffer cells; thus they are critical in the apoptotic and/or necrotic cell death of hepatocytes and sinusoidal endothelial cells.[1,8]

Antioxidant treatments have been shown to be effective therapeutic approaches for the prevention of hepatic I/R injury in animal experiments.[4] SOD is the antioxidant enzyme that catalyzes the dismutation of O_2^- to O_2 and the less-reactive species, H_2O_2. There are 3 isoforms of SOD: copper/zinc SOD (Cu/Zn-SOD), manganese-SOD, and extracellular SOD (EC-SOD). Cu/Zn-SOD is localized in the cytosol and nucleus of all cell types and plays a major role in the intracellular antioxidative system. Manganese-SOD is exclusively localized in the mitochondria. The unique extracellular distribution and secretory nature of EC-SOD confer antioxidative protection against ROS released into the extracellular space.[10] Moreover, O_2^- existing in the extracellular space is unable to cross the cell membrane, to be removed by intracellular SOD.[11] Providing more SOD activity by delivering the enzyme or SOD mimics has been shown to be effective in preventing a variety of types of liver injury. However, the half-life of SOD is only 6 minutes.[12] Therefore, a gene therapy strategy aimed at increasing levels of SOD that provides sufficient gene expression in the donor liver is desirable for protection against hepatic I/R injury. Our previous studies, which employed polylipid nanoparticles (PLNP) to deliver the human EC-SOD gene in a mouse model of acute liver injury caused by D-galactosamine plus lipopolysaccharide, demonstrate the effectiveness of this gene transfer approach to the liver, and the protection of EC-SOD overexpression against acute toxicity.[13]

Catalase is a potent scavenger of H_2O_2, and provides another means of inhibiting oxidant stress. It prevents the formation of more toxic HO^- when SOD is insufficient to remove O_2^- overload. Thus, the production of catalase provides additional antioxidative activity against oxidative stress that is present during the I/R procedure. The delivery of SOD and catalase proteins has successfully prevented hepatic I/R injury in mice.[14,15] However, to our knowledge there has been no animal study that assesses the efficacy of EC-SOD and/or catalase gene transfer on hepatic I/R injury. In this study we report the effectiveness of EC-SOD and catalase gene delivery by our PLNP in preventing subsequent warm hepatic I/R injury in mice.

MATERIALS AND METHODS

Subcloning EC-SOD and Catalase Plasmids

The pEGFP-C1-ECSOD plasmid was generated as described previously.[13] The human catalase gene sequence was obtained by digestion with restriction endonucleases Sal1 and Xba1 from the pC1-catalase plasmid (kindly provided by Dr. Arthur Cederbaum, Mount Sinai School of Medicine, New York, NY). The sequence fragment was blunted at both ends, and then a Sal1 linker was added to the 3′ end. The resulting sequence was further digested with HindIII, and the product was subcloned into an expression vector, pEGFP-C1, at the HindIII and Sal1 sites by replacing the EGFP sequence to form pC1-catalase. The sequence of the new plasmid was verified by sequencing of the polymerase chain reaction product. The resulting plasmid deoxyribonucleic acid (DNA) was amplified, extracted, purified, and quantitated as described previously.[13,16] AQ: 1 AQ: 1 AQ: 1 AQ: 1 AQ: 1

Generation and Size Measurement of PLNP AQ: 3

Polycationic lipid was synthesized and validated as previously described[17] and PLNP were formulated from polycationic lipid and cholesterol in a molar ratio of 3:1 (polycationic lipid to cholesterol). The size of PLNP at different times of generation was between 200 and 250 nm before use. For animal experiments, PLNP were complexed with plasmid DNA to form polyplexes at a charge ratio of 5:1. For each mouse, 200 μL of PLNP suspension containing 0.3 μmol polycationic lipid, 0.1 μmol cholesterol and 100 μg plasmid DNA was injected via the portal vein.

Mouse Model of Warm Hepatic I/R Injury and Experimental Design

ICR mice (from Charles River Laboratory, Wilmington, MA) were fed a pellet diet and water ad libitum, and kept on a 12-hour-light/dark cycle. The animal experiment was performed according to a protocol approved by the University of California Davis Institutional Animal Care and Use Committee. The protocol was prepared in accordance with the National Institutes of Health Animal Guidelines. PLNP-mediated EC-SOD and/or catalase gene delivery to mouse liver was conducted according to our previous description.[13] Animals were randomly divided into groups as listed in Table 1 and all received 1 injection of triiodothyronine (4 mg/kg subcutaneously) 1 day before the injection of polyplexes in order to stimulate hepatocyte proliferation.[16] The time line of the experiment is shown in Illustration 1. AQ: 1 T1 AQ: 4 AQ: 1 F8

One day after triiodothyronine injection, a surgical procedure was undertaken to inject polyplexes through the portal vein. The total volume of 300 μL of polyplexes (control, EC-SOD, catalase, or EC-SOD/catalase polyplexes) consists of a mixture of 200 μL of PLNP and 100 μg of plasmid DNA (pEGFP-C1, pEGFP-C1-ECSOD, or pC1-catalase). For the EC-SOD/catalase polyplex-injected group, 50 μg of each plasmid DNA was used. At 1

TABLE 1. Experimental Design of Warm Hepatic I/R Injury

Group	T_3 (4 mg/kg, s.c.)	N.S.	Control polyplexes (pEGFP-C1)	EC-SOD polyplexes (pEGFP-C1-ECSOD)	Catalase polyplexes (pC1-catalase)	I/R procedure
A	+	300 μL	−	−	−	+
B	+	−	+ (100 μg DNA)	−	−	+
C	+	−	−	+ (100 μg DNA)	−	+
D	+	−	−	−	+ (100 μg DNA)	+
E	+	−	−	+ (50 μg DNA)	+ (50 μg DNA)	+

Abbreviations: T_3, triiodothyronine; s.c., subcutaneous; pEGFP-C1, _____; pC1, _____.

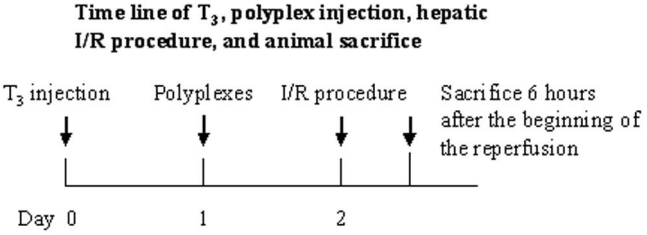


Illustration 1. Time line of triiodothyronine (T_3), polyplex injection, hepatic I/R procedure, and animal sacrifice.

day after polyplex injection, the mice were operated on again for an I/R procedure.[15] In brief, mice were anesthetized with pentobarbital sodium (60 mg/kg intraperitoneally). Laparotomy was performed with a midline incision. Following surgical exposure of the portal vein, mice were injected with heparin (100 unit/kg) intravenously to prevent the formation of blood clots during ischemia. The portal vein and hepatic artery were occluded for 30 minutes with a microaneurysm clamp to induce hepatic ischemia. Then, the clamp was removed to allow reperfusion of the liver. A sham surgery was performed in heparinized animals under anesthesia but without occluding the vessels.

At 2 and 6 hours after starting the reperfusion, blood samples were collected via tail incision or from the vena cava before sacrifice. Portions of the liver tissue were fixed in 10% neutralized formalin for histological evaluation or snap frozen in liquid nitrogen and maintained at −80°C until homogenization for the various biochemical assays.

Serum Alanine Aminotransferase Assay

Serum alanine aminotransferase (ALT) levels serving as an indicator of liver injury were analyzed using a diagnostic kit (Sigma Chemical, St. Louis, MO), and expressed as units per milliliter.

Measurement of ROS Generation in Liver Homogenates

2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo [1,2-α] pyrazin-3-one (MCLA)-enhanced chemiluminescence was used to determine O_2^-·generation. On the day of measurement, approximately 10 mg of frozen liver tissue was homogenized on ice in 1 mL of homogenization buffer containing 20 mmol/L 4-2-hydroxyethyl-1-piperazineethanesulfonic acid and 10 mmol/L ethylene diamine tetraacetic acid. The homogenate was subjected to low speed centrifugation (1,000*g*) for 10 minutes to remove debris. Luminometer vials containing 2 mL of prewarmed Krebs-4-2-hydroxyethyl-1-piperazineethanesulfonic acid buffer with 1.0 μmol/L of MCLA were placed in the dark for at least 20 minutes. After the dark adaptation, background readings were recorded in a luminometer (Lumat LB; Berthold Technologies, BmbH & Co. KG, Germany), and then 20 μL of homogenate supernatant was added to a vial containing MCLA. Chemiluminescent emission in relative light units was recorded during a plateau phase of each recording period, corrected by subtracting the background reading. The calculated value was used to express the integrated values of chemiluminescence (relative light units/second/μg protein).

Assays of Liver Glutathione, Malondialdehyde, and Hydrogen Peroxide

Levels of the reduced form of liver glutathione (GSH) and malondialdehyde (MDA) in the liver tissue were measured spectrophotometrically 6 hours after starting reperfusion by commercially available kits (OXIS Research, Portland, OR). The levels of GSH and MDA are expressed as nanomoles per milligram of protein. Liver H_2O_2 levels were assayed with an Amplex Red kit from Invitrogen (Carlsbad, CA) according to the manufacturer's instruction and expressed as millimoles per milligrams of tissue.[18]

Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction for EC-SOD Gene Expression

Real-time quantitative reverse transcriptase polymerase chain reaction was employed to assess the human EC-SOD gene expression levels in mouse liver using mouse β-actin as a housekeeping gene control, as reported previously.[13,19] The relative gene expression in different groups was calculated based on the average level of the normal saline (N.S.) control group.

Western Blot Analysis for Catalase Protein Levels

Western blotting was used to confirm recombinant human catalase protein expression in the liver with a method reported previously[20] using sheep anti-human catalase polyclonal primary antibodies (Abcam, Cambridge, MA).

Measurement of SOD and Catalase Activity

SOD and catalase activity in liver homogenates was measured with the CalBiochem SOD Assay Kit and Catalase Assay Kit (CalBiochem, San Diego, CA). The SOD activity was expressed as units per milligrams of protein. The catalase activity was expressed as micromoles of H_2O_2 per minute per milligrams of protein.

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay

Nuclear protein was prepared as reported previously[21] and used for electrophoretic mobility shift assay. AP-1 and nuclear factor-κB (NF-κB) DNA binding activity was determined by electrophoretic mobility shift assay (Pierce, Rockford, IL). For confirming the specificity of binding reactions, antibodies against NF-κB subunits p65 and p50, or c-Jun and c-Fos, or unlabeled cold probe were incubated with nuclear protein before the addition of biotin-end-labeled probes for the binding reaction.[22]

Histological Examination

Fixed liver specimens were embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin. Micrographs were taken with a digital camera. The extent of hepatocellular death was semiquantitatively scored by a pathologist blinded to the experimental protocol, according to a modified scoring system of Batts and Ludwig.[23]

Determination of Serum Tumor Necrosis Factor-α Levels

Serum tumor necrosis factor-α (TNF-α) levels after the I/R procedure were determined with an enzyme-linked immunosorbent assay kit (ELISA kit; R&D Systems, Minneapolis, MN) and expressed as nanograms per milliliter.

Statistical Analysis

Most data were expressed as means ± standard error of the mean and evaluated with analysis of variance and Newman-Keuls test for multiple comparisons among groups. Wilcoxon signed rank test was employed for a semiquantitative score of liver injury, followed by *q* tests for multiple comparisons among groups. $P < 0.05$ was considered statistically significant.

RESULTS

Overexpression of Antioxidative Genes in Mouse Livers

We delivered EC-SOD and/or catalase genes into mouse livers using our PLNP with a prior injection of triiodothyronine. As shown in Figure 1A, there was an approximately 10-fold increase in liver SOD activity in mice receiving either EC-SOD or EC-SOD plus catalase polyplex injection via the portal vein 30 hours after the injection. Surprisingly, catalase polyplex injection resulted in a 5.5-fold increase in SOD activity. Real-time reverse transcriptase polymerase chain reaction showed that EC-SOD messenger ribonucleic acid levels increased approximately 50.8-fold and 22.9-fold in animals receiving EC-SOD or EC-SOD plus catalase gene delivery when compared to those receiving the control polyplex injection (Fig. 1B). Catalase polyplex injection did not significantly affect EC-SOD gene expression levels. Thus, it seems that the enhanced SOD activity in catalase polyplex-injected mice may be associated with posttranscriptional modification or alteration in activities of EC-SOD or other SOD isoforms.

Catalase polyplex injection led to an approximately 10-fold increase in liver catalase activity (Fig. 2A), and EC-SOD gene delivery enhanced catalase activity approximately 6.6-fold when compared to the control polyplex injection. The liver catalase activity in the animals receiving EC-SOD plus catalase gene delivery was increased 8-fold. Western blot analysis indicated that catalase protein levels were markedly elevated in the liver of mice receiving catalase gene delivery, but not in those with EC-SOD gene delivery. Thus, it is evident that our PLNP-mediated human EC-SOD and catalase gene delivery resulted in marked overexpression of these 2 antioxidative genes and a profound elevation in enzyme activity in the mouse livers.

Enhanced Superoxide Anion Release and H_2O_2 Production During Hepatic I/R

No direct evidence exists that there is enhanced generation of $O_2^{-\cdot}$ and related ROS in rodent models of hepatic I/R. We employed MCLA, which is proportional to levels of $O_2^{-\cdot}$ and singlet oxygen, to investigate whether the I/R procedure enhanced the chemiluminescent emission from the liver homogenates. We found that light emission from the liver homogenates started to increase at 5 minutes, was significantly elevated at 2 hours, and was 4.9-fold higher 6 hours after starting the reperfusion, compared to animals with sham surgery. The enhanced chemiluminescent emission caused by the I/R procedure was almost completely inhibited by prior EC-SOD or catalase polyplex injection (Fig. 3). This finding provides convincing evidence that enhanced SOD and catalase activity, as the result of the gene delivery, effectively eliminated the oxidative stress during the I/R procedure. We also measured liver H_2O_2 levels with a highly sensitive fluorescent probe, Amplex Red reagent, and found that H_2O_2 levels were significantly higher at 5 minutes after reperfusion

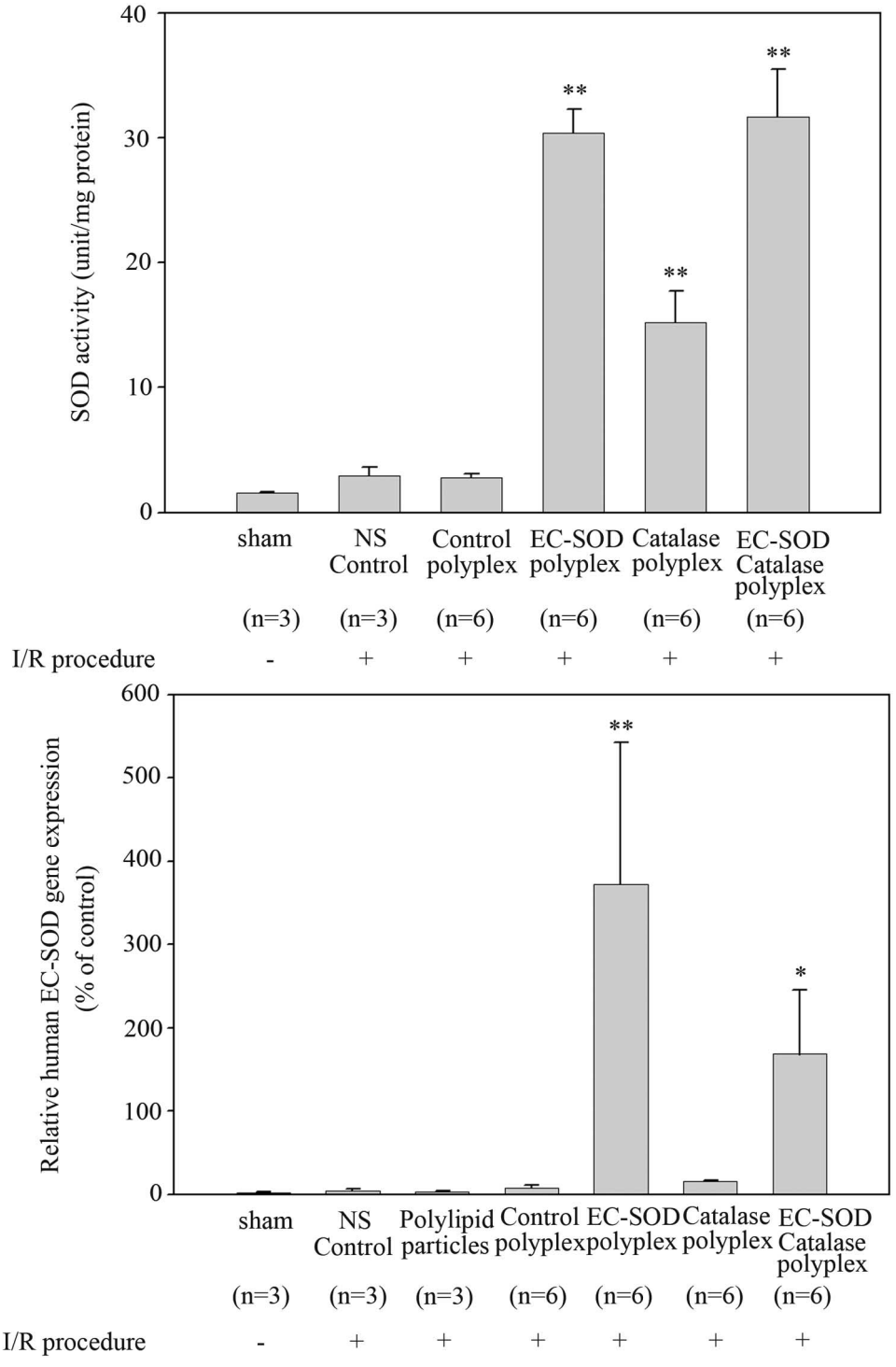


Figure 1. Human EC-SOD gene expression and SOD activity in liver tissue of mice receiving PLNP-mediated EC-SOD gene delivery. (A) Liver SOD activity after PLNP-mediated EC-SOD gene delivery and subsequent I/R-induced liver injury. Total SOD activity in liver tissue was measured spectrophotometrically 30 hours after portal vein injection of polyplexes. (B) Human EC-SOD messenger ribonucleic acid levels in mouse liver tissue were determined with real-time quantitative reverse transcriptase polymerase chain reaction using mouse β-actin as a housekeeping gene control. The relative human EC-SOD gene expression levels in 5 other groups were calculated based on an average level in the N.S. control group. In both panels, *P < 0.05, **P < 0.01 compared with N.S. control, sham-operated, or control polyplex groups. Groups of animals which received the I/R procedure are indicated.

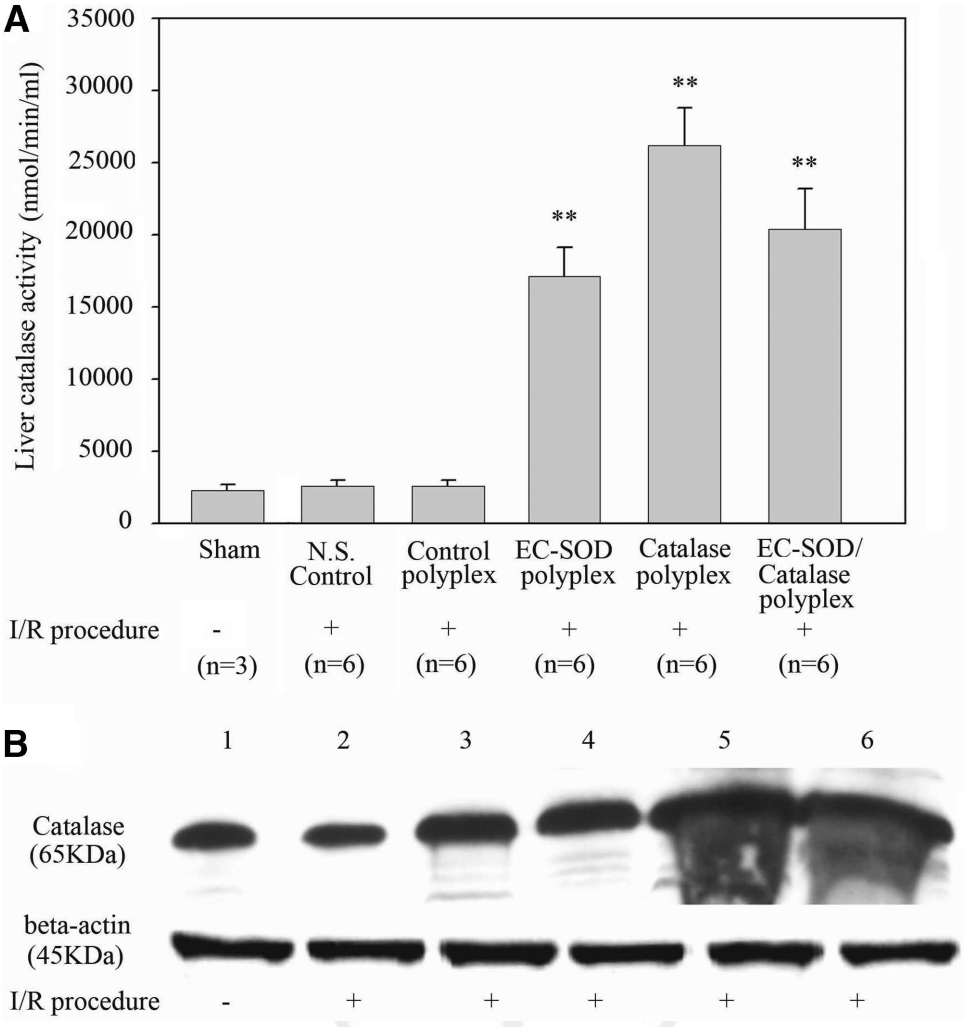


Figure 2. Liver catalase protein levels and catalase activity in mice receiving PLNP-mediated human catalase gene delivery. (A) Liver catalase activity in mice after PLNP-mediated human catalase gene delivery and subsequent I/R-induced liver injury. Liver catalase activity was measured spectrophotometrically 30 hours after portal vein injection of polyplexes. *$P < 0.05$, **$P < 0.01$ compared to N.S. control, sham-operated, or control polyplex groups. (B) Liver human catalase protein levels were determined by Western blot analysis using polyclonal antibodies against human catalase. β-actin was used as a loading control. Lane 1: sham-operated; Lane 2: I/R procedure plus N.S. control; Lane 3: I/R procedure plus control polyplexes; Lane 4: I/R procedure plus EC-SOD polyplexes; Lane 5: I/R procedure plus catalase polyplexes; and Lane 6: I/R procedure plus EC-SOD/catalase polyplexes.

compared to normal liver tissue, and were maintained at a high level for 6 hours (Fig. 4A). This level was not changed in the I/R procedure plus N.S. control or control polyplexes, but was markedly reduced by either EC-SOD, catalase, or the combination of the 2-gene delivery (Fig. 4B).

Amelioration of I/R-induced Acute Liver Injury

As shown in Figure 5, the I/R procedure caused a marked increase in serum ALT levels during the first 2 to 6 hours after starting the reperfusion. Prior intravenous injection of either EC-SOD or catalase polyplexes via the portal vein resulted in a marked decrease in serum ALT levels compared to animals with I/R plus N.S. injection (at the 6-hour point, $P < 0.01$). It appeared that the combination of EC-SOD and catalase polyplexes tended to exert further improved protection than either EC-SOD or catalase polyplexes alone; however, the differences were not statistically significant at all time points. For controls, additional animals were injected with PLNP alone or control polyplexes without the I/R procedure. At 1 day after the injection in animals that did not have the I/R procedure, serum ALT levels in animals receiving PLNP alone (45 ± 7 units/mL, n = 3) or control polyplexes (57 ± 6.5 units/mL, n = 3) were similar to levels in saline controls (42 ± 8.5 units/mL, n = 3). The data indicate that neither PLNP nor the control polyplexes are toxic to the liver.

The extent of hepatocellular death in liver sections was assessed by a pathologist blinded to the experimental protocol (Table 2 and Fig. 5B). Liver sections from normal or sham-operated mice showed no necrosis. The I/R procedure led to marked necrosis in the central lobule (Zone III) with significant inflammatory

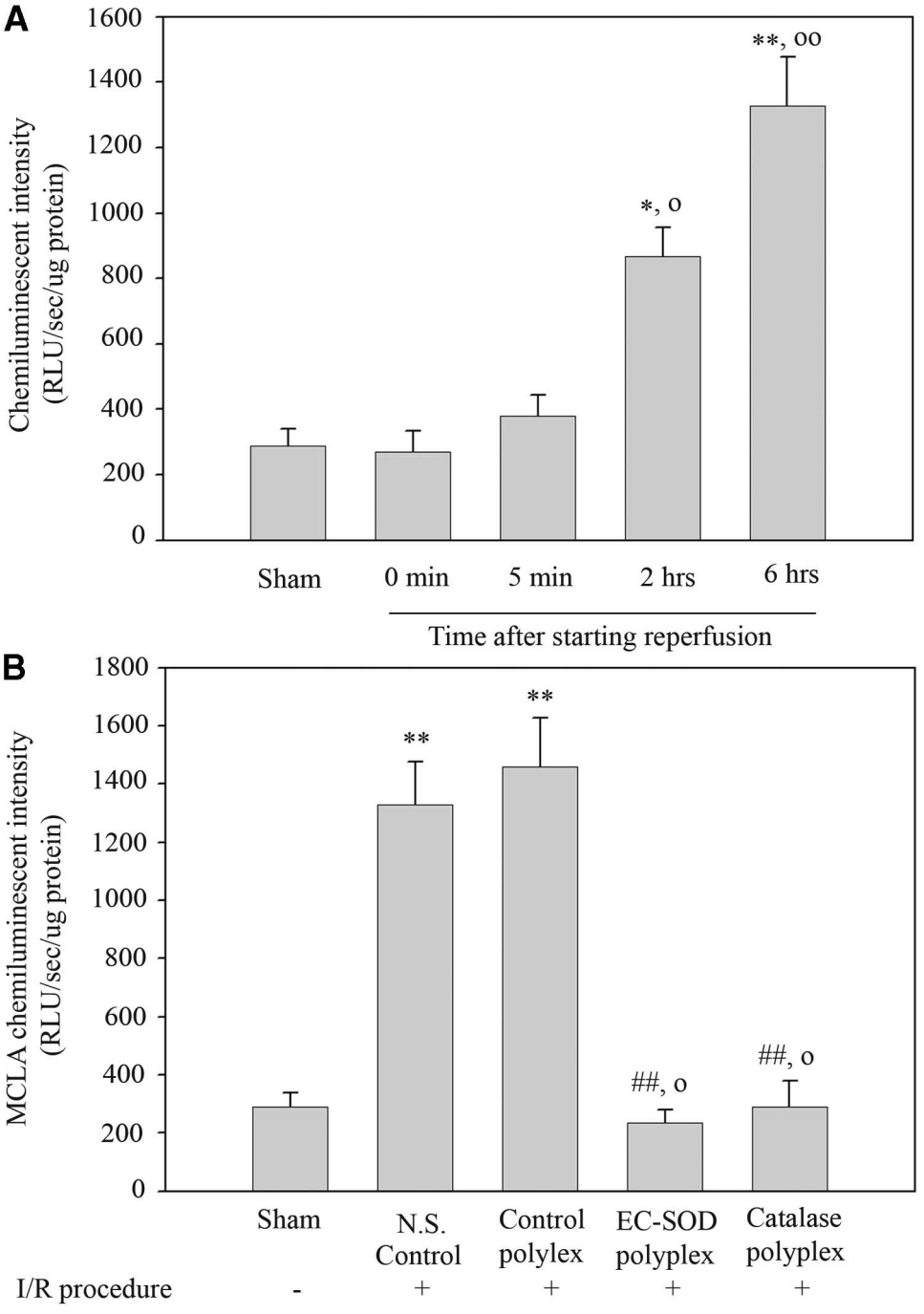


Figure 3. I/R-induced superoxide anion production and the effects of antioxidative enzyme gene delivery in mouse liver. (A) The superoxide anion production was determined in mouse liver homogenates with a superoxide anion tracer, MCLA, and the MCLA chemiluminescent emission was recorded in a luminometer and expressed as relative light units per second per micrograms of protein. *P <0.05, **P < 0.01 compared to sham-operated or 0 minutes after the completion of ischemia. $^{\circ}P$ < 0.05, $^{\circ\circ}P$ <0.01 compared to 5 minutes after the beginning of reperfusion procedure. (B) The effects of antioxidative gene delivery on superoxide anion production during the I/R procedure. The MCLA chemiluminescent signal was determined 6 hours after starting the reperfusion. **P < 0.01 compared to sham-operated control. $^{\#\#}P$ < 0.01 compared to N.S. control or control polyplex plus I/R procedure (n = 6 in both (A) and (B)).

infiltration (Fig. 5Ba). Liver histology revealed a similar degree of massive cell death and inflammatory infiltration in I/R-challenged animals plus portal vein injection of N.S. (Fig. 5Bb) or control polyplexes (Fig. 5Bc). A significant reduction in necrosis was found in the liver of animals receiving injection of EC-SOD, catalase, or EC-SOD/catalase polyplexes (Fig. 5Bd, Be, and Bf), compared to the control groups (Fig. 5Ba, Bb, and Bc). The degree of necrosis, as judged by a histological semi-quantitative score which is shown in Table 2, verifies

tapraid5/zlt-liver/zlt-liver/zlt00107/zlt1119d07g | heckt | S=5 | 10/20/06 | 9:18 | Art: 06-320

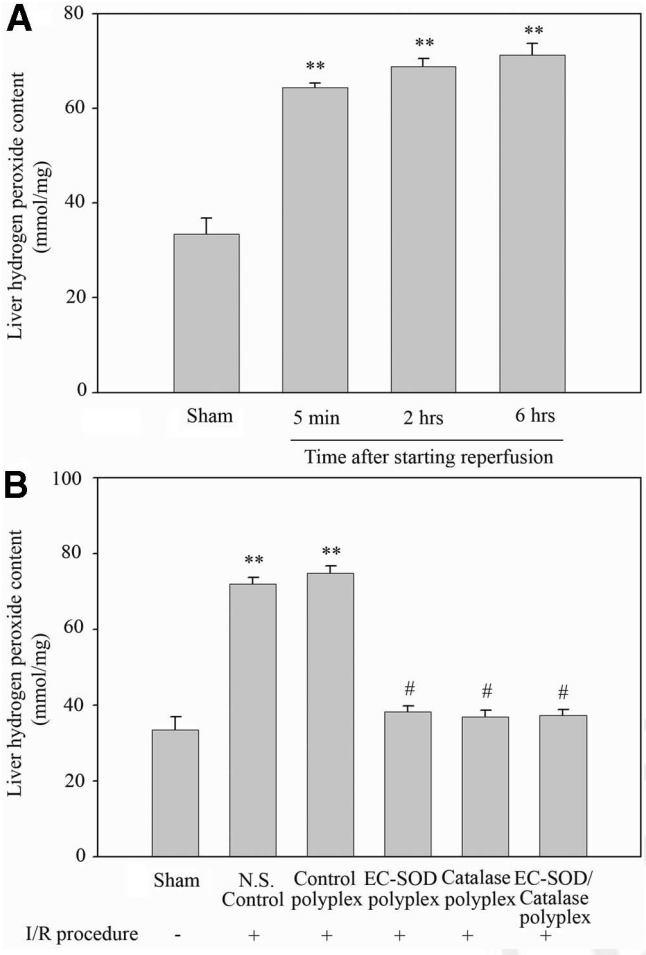


Figure 4. Liver hydrogen peroxide levels after the I/R procedure and antioxidative gene delivery. Liver hydrogen peroxide (H_2O_2) was determined by an Amplex Red reagent with a kit (Invitrogen) according to the manufacturer's instructions, and expressed as millimoles per milligram of tissue. **$P < 0.01$ compared to the sham-operated group and $^{\#}P < 0.05$ compared to the I/R procedure plus N.S. control or control polyplexes (n = 4).

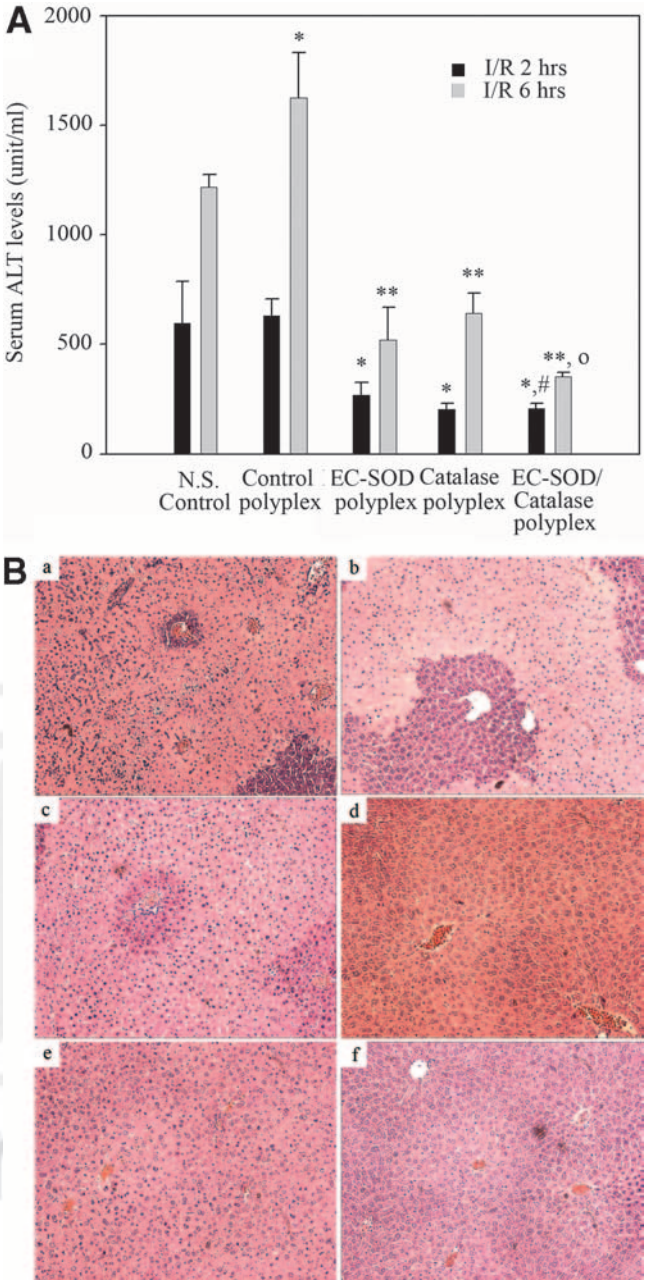


Figure 5. The attenuation of acute liver injury caused by the I/R procedure with antioxidative gene delivery. (A) Serum ALT levels in mice receiving antioxidative gene delivery and subsequent I/R procedure. Serum ALT levels were determined 2 and 6 hours after starting reperfusion (n = 6 in each group). *$P < 0.05$, **$P < 0.01$ compared to I/R procedure plus N.S. control; $^{\#}P < 0.05$ compared to the I/R procedure plus EC-SOD polyplex at 2-hour time point, $^{\circ}P < 0.05$ compared to I/R procedure plus catalase polyplex group at 6-hour time point. (B) Micrographs of liver histology of I/R-induced acute liver injury and the protection of antioxidative gene delivery. (a) I/R-induced acute liver injury 6 hours after starting reperfusion; (b) I/R-induced acute liver injury plus N.S. injection; (c) I/R-induced acute liver injury plus control polyplex injection; (d-f) attenuated liver injury with EC-SOD (d), catalase (e), and combined EC-SOD with catalase (f) gene delivery. Magnification 100×.

COLOR

that liver injury in mice receiving EC-SOD, catalase, or EC-SOD/catalase polyplexes was markedly attenuated when compared to those treated with N.S. control (Fig. 5Bb) or control polyplexes (Fig. 5Bc).

Restored GSH and Reduced MDA Levels by EC-SOD and/or Catalase Gene Delivery

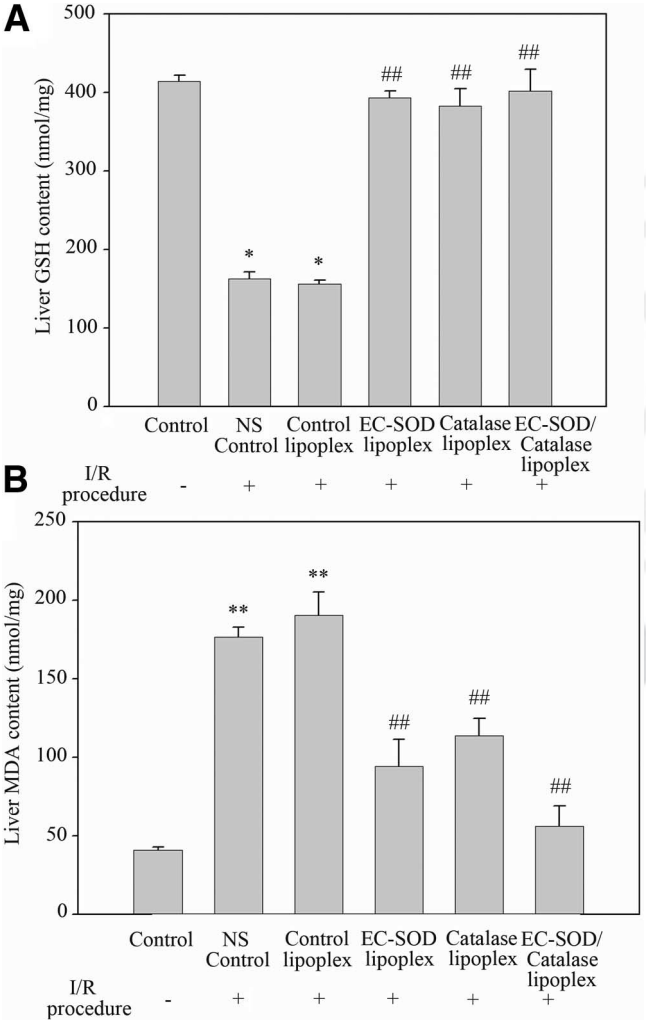
Liver GSH and MDA levels were determined as indicators of oxidant stress and lipid peroxidation during the hepatic I/R procedure. As shown in Figure 6, the antioxidative gene delivery led to a restoration of decreased GSH levels and a reversion of elevated MDA levels in the liver, and the levels were close to controls. Animals receiving injection of EC-SOD and/or catalase polyplexes also had preserved GSH levels and reversed the increased liver MDA levels in comparison with those receiving injection of N.S. or control polyplexes (Fig. 6A and B). Both liver GSH and MDA levels in animals

F6

TABLE 2. Semiquantitative Score of Hepatocellular Injury

Score	I/R procedure + N.S. control	I/R procedure + control polyplexes	I/R procedure + EC-SOD polyplexes	I/R procedure + catalase polyplexes	I/R procedure + EC-SOD/ catalase polyplexes
0	0	0	4	3	3
1	1	1	1	0	2
2	2	1	0	2	1
3	3	4	1	1	0
P value	N/A	N/A	<0.05*	<0.05*	<0.05*

Abbreviation: N/A, not applicable.
*Compared with I/R procedure plus control polyplex or N.S. control groups.



F7

Figure 6. (A) Liver GSH and (B) MDA levels in mice with I/R procedure plus antioxidative gene delivery. Liver GSH and MDA content was determined spectrophotometrically and expressed as nanomoles per milligram of protein of the tissue. **$P < 0.01$ compared with normal controls without I/R procedure. \#$P < 0.05$, \#\#$P < 0.01$ compared to N.S. control or control polyplexes plus I/R procedure (n = 4; except in the control group n = 3).

receiving EC-SOD or catalase or their combination gene delivery were close to those in controls without the I/R insult.

Activation of NF-κB or AP-1 During the I/R Procedure

NF-κB and AP-1 activation may lead to the coordinated expression of many genes that are involved in the mediation and perpetuation of the inflammatory response. We were therefore interested in the effects of EC-SOD, catalase, or their combined gene delivery on NF-κB and AP-1 activation during the hepatic I/R injury. As shown in Figure 7Aa and Ab, the I/R procedure significantly enhanced DNA binding activity of NF-κB and AP-1 as compared to the sham-operated group. The enhanced DNA binding activity caused by the I/R procedure was markedly inhibited by prior EC-SOD or catalase gene delivery, which was confirmed by the antibody or unlabeled probe blocking experiments (Fig. 7A). Neither N.S. nor the control polyplexes had appreciable effects on DNA binding activity of NF-κB and AP-1 as compared to the sham surgery. Serum TNF-α levels increased as early as 2 hours after starting reperfusion, and continued to increase up to 6 hours after the reperfusion (Fig. 7B). The antioxidative gene delivery completely prevented the increase in serum TNF-α levels, and TNF-α levels in EC-SOD, catalase, or EC-SOD/catalase polyplexes plus the I/R procedure groups were similar to the sham-operated controls without the I/R procedure.

DISCUSSION

An I/R process is inevitable in organ transplantation. The extent of I/R-associated liver injury is one of the major factors directly affecting the graft survival and function after transplantation. The injury is characterized by early necrosis due to hypoxia and mitochondrial malfunction during an extended period of ischemia, marked apoptosis during reperfusion due to the activation of infiltrating neutrophils and resident Kupffer cells, and release of ROS from various sources.[24,25] Thus, enhancing antioxidative gene delivery is 1 of molecular intervention with an obvious rationale, in addition to other gene therapy approaches.[26-28] In the

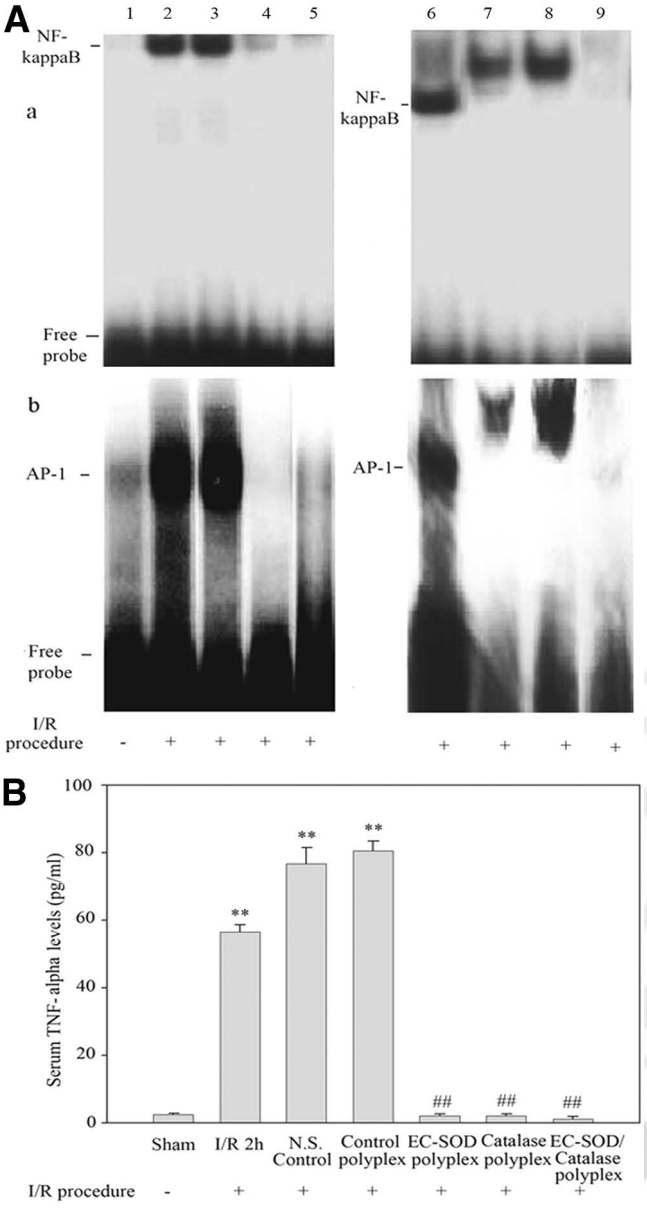


Figure 7. I/R-induced NF-κB and AP-1 activation, and the effects of antioxidative gene delivery. (A) DNA binding of NF-κB and AP-1 was determined by electrophoretic mobility shift assay after sham operation or 6 hours following I/R procedure. Representative images from at least 3 independent experiments. In both (a) and (b): Lane 1: Sham-operated; Lane 2: I/R procedure plus N.S. control; Lane 3: I/R procedure plus control polyplexes; Lane 4: I/R procedure plus catalase polyplexes; Lane 5: I/R procedure plus EC-SOD polyplexes; and Lane 6: nuclear protein samples from animals with the hepatic I/R procedure. Supershift assays using specific antibodies against p50 (lane 7) and p65 (lane 8) subunits in (a), and c-Jun (lane 7), and c-Fos (lane 8) in (b) are also shown. The specificity of the binding reaction was also examined by the inclusion of unlabeled cold probe (lane 9) in both panels. (B) Serum TNF-α levels as measured by an enzyme-linked immunosorbent assay kit (R&D Systems) in mice with hepatic I/R-induced liver injury. **$P < 0.01$ compared to normal controls, $^{\#\#}P < 0.01$ compared to I/R plus N.S. or control polyplex injection.

present study, we explored a new application of nonviral PLNP in the mediation of antioxidative gene delivery for the prevention of I/R-induced liver injury. Thus, the study evaluated the potential of this nonviral gene transfer approach for organ transplantation.

One characteristic of I/R-induced liver injury is enhanced ROS release during the procedure. Most studies have suggested that the O_2^- are the primary oxidant species generated during the I/R procedure,[4] and that O_2^- may be capable of directly or indirectly mediating a substantial portion of postischemic injury. MCLA has been used to detect O_2^- levels in Kupffer cells.[29] Due to a very short half-life and the instability of O_2^-, no convenient and reliable method is available for its in vivo detection. For this purpose, a specific charged-coupled device camera is needed to couple with a microscopic objective lens. This allows for direct contact with the tissue, monitoring chemiluminescent signals while the organ is being perfused.[30,31] The system cannot be readily applied to liver injury model systems due to the limitation in the detection of signal depth within the tissue and the duration of monitoring. In the present study, we adapted a method for other systems,[32] and added MCLA as the substrate to liver homogenates immediately after completing the I/R procedure. We determined the chemiluminescent emission in order to reflect the generation of O_2^- in liver homogenates. The results showed that the increase of chemiluminescent emission started 5 minutes after beginning of the reperfusion, and that a significant increase in O_2^- generation and other ROS levels in the liver was documented after 2 to 6 hours of the reperfusion. To our knowledge, this is the first direct evidence that during the reperfusion phase there is a marked increase in O_2^- release in the liver. This finding further provides the rationale for antioxidative treatment in the prevention of I/R-induced liver injury.

SOD and catalase are 2 critical enzymes in the antioxidative defense. Early work involving the direct administration of recombinant SOD or catalase protein through the tail vein eliminated excessive ROS and decreased neutrophil accumulation in the liver.[15] However, the therapeutic effects of the recombinant proteins are limited due to low tissue availability and very short half-life.[15] Adenoviral vector-mediated Cu/Zn-SOD protected mice from I/R-induced acute liver injury, but an adenoviral vector containing EC-SOD was not effective due to no increase in SOD activity in the liver and only a slight increase in serum.[33] A further increase in adenoviral titer led to a 3-fold increase in liver SOD activity and protected the injury.[33] It is known that a high titer of adenoviruses may cause direct damage to the liver, and that the occurrence of immune responses against adenoviral components impedes the wide application of this viral vector.[34] We used an established PLNP formulation[16] to deliver both EC-SOD and catalase genes to the mouse liver, and achieved much higher levels of SOD activity than reported by Wheeler et al.,[33] as well as a high level of catalase activity in mouse liver. The overexpression of these 2 antioxidative genes significantly suppressed the

subsequent I/R-induced acute liver injury as reflected by a marked reduction of serum ALT levels, and improved liver histology. Moreover, PLNP-mediated EC-SOD and/or catalase gene delivery completely suppressed the increased $O_2^{-\cdot}$ levels and H_2O_2 production caused by the I/R procedure, and significantly attenuated the oxidative stress as evidenced by lower MDA levels and restored GSH levels.

A significantly lower level of serum ALT was noted at 6 hours of reperfusion in the combination of EC-SOD with catalase gene delivery when compared to either EC-SOD or catalase gene delivery alone (Fig. 4). This suggests synergetic protection by these 2 antioxidative enzymes in this hepatic I/R model system because only half the amount of each plasmid was used. This is not unexpected because each enzyme targets a complementary antioxidative system. In our previous studies[13] we found that increased SOD activity was detected in both the extracellular space (culture medium) and cell lysates after transfection of EC-SOD plasmid in Hep G_2 cells, and that catalase acts primarily on intracellular H_2O_2 (increased catalase activity was only detected in cell lysates after transduction of a lentiviral catalase vector in Hep G_2 cells, our unpublished results). Therefore, it appears that the combined gene delivery led to enhanced antioxidative protection in both the intracellular and extracellular space. We injected thyroid hormone (triiodothyronine) prior to polyplex injection in order to promote hepatocyte proliferation and to enhance gene transfer efficacy, as we reported previously.[13,16] Taken together, our PLNP and the gene delivery approach are very useful in delivering antioxidative genes for the prevention of acute I/R-induced liver injury. To our knowledge, this is the first evidence that a nonviral gene transfer agent has successfully delivered functional genes to improve graft function and survival. Thus, this nonviral gene delivery approach has the potential to be clinically applicable during the window of organ harvest, preservation, and transplantation.

In the present study, we also observed that the gene transfer of catalase alone significantly increased the SOD activity in the mouse liver, but did not significantly affect EC-SOD messenger ribonucleic acid expression when compared to control animals. Moreover, the delivery of the catalase gene markedly suppressed O_2^- production (Fig. 3B), which may be explained through enhanced SOD activity. In addition, SOD gene delivery enhanced catalase activity, and reduced liver H_2O_2 levels during the I/R procedure. The mechanisms that explain how human catalase gene delivery enhances SOD activity in the liver, and vice versa, are unclear. It is tempting to speculate that higher SOD activity resulting from the gene transfer will generate more H_2O_2 from O_2^-, and a high substrate level of H_2O_2 in turn elicits a compensatory response involving the induction of catalase expression in the liver. In a study by Dai et al.,[35] elevated catalase activity was noted in joint fluid after EC-SOD gene delivery, and vice versa; catalase gene delivery also increased SOD activity, but neither reached statistical significance.

Another important finding in this study is that both EC-SOD and catalase gene expression suppressed the activation of NF-κB and AP-1 caused by the I/R procedure. The activation of these 2 transcription factors has been strongly implicated as a mechanism in hepatic I/R injury and inflammation.[36,37] and it is consistent with elevated serum levels of TNF-α, which contributes to hepatic I/R injury via the extrinsic pathway.[25,38] The downstream effects of the activation of these transcription factors during the I/R procedure and the effects of gene transfer of the antioxidative enzymes on this process will be the focus of further investigation.

In conclusion, the findings in the present study demonstrate that PLNP-mediated EC-SOD or catalase gene delivery resulted in high levels of the transgene activity in the liver, and markedly attenuated liver I/R injury. The protection is associated with enhanced antioxidative effects in the liver. To our knowledge, this is the first successful demonstration that PLNP-mediated EC-SOD and/or catalase gene delivery ameliorates warm I/R-induced liver injury in mice. This approach may become a potential therapy to improve the graft function and survival of liver transplantation.

ACKNOWLEDGMENTS

We thank Mr. Christopher Bishop, Ms. Lauren Eisenbud, and Andreea Catana, MD for their technical assistance.

REFERENCES

1. Teoh N, Farrell GC. Hepatic ischemia reperfusion injury: pathogenic mechanisms and basis for hepatoprotection. J Gastroenterol Hepatol 2003;18:891-902.
2. Fan C, Zwacka RM, Engelhardt JF. Therapeutic approaches for ischemia/reperfusion injury in the liver. J Mol Med 1999;77:577-592.
3. Hines IN, Harada H, Wolf R, Grisham MB. Superoxide and post-ischemic liver injury: potential therapeutic target for liver transplantation. Curr Med Chem 2003;10:2661-2667.
4. Selzner N, Rudiger H, Graf R, Clavien PA. Protective strategies against ischemic injury of the liver. Gastroenterology 2003;125:917-936.
5. Karwinski W, Soreide O. Allopurinol improves scavenging ability of the liver after ischemia/reperfusion injury. Liver 1997;17:139-143.
6. Hines IN, Hoffman JM, Scheerens H, Day BJ, Harada H, Pavlick KP, et al. Regulation of postischemic liver injury following different duration of ischemia. Am J Physiol 2003;284:G536–G545.
7. Arteel GE. Oxidants and antioxidants in alcohol-induced liver disease. Gastroenterology 2003;124:776-790.
8. Jaeschke H, Lemaster JJ. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. Gastroenterology 2003;125:1246-1257.
9. Baliley SM, Reinke LA. Antioxidants and gadolinium chloride attenuate hepatic parenchymal and endothelial cell injury induced by low flow ischemia and reperfusion in perfused rat livers. Free Radic Res 2000;32:497-506.
10. Marklund SL. Extracellular superoxide dismutase. Methods Enzymol 2002;349:74-80.
11. Winterbourn CC, Stern A. Human red cells scavenge extracellular hydrogen peroxide and inhibit formation of hypochlorous acid and hydroxyl radical. J Clin Invest 1987; 80:1486-1491.

12. Nguyen WD, Kim DH, Alam HB, Provido HS, Kirkpatrick JR. Polyethylene glycol-superoxide dismutase inhibits lipid peroxidation in hepatic ischemia/reperfusion injury. Crit Care 1999;3:127-130.
13. Wu J, Liu L, Yen RD, Catana A, Nantz MH, Zern MA. Liposome-mediated extracellular superoxide dismutase gene delivery protects against acute liver injury in mice. Hepatology 2004;40:195-204.
14. Yabe Y, Koyama Y, Nishikawa M, Takakura Y, Hashida M. Hepatocyte-specific distribution of catalase and its inhibitory effect on hepatic ischemia/reperfusion injury in mice. Free Rad Res 1999;30:265-274.
15. Yabe Y, Kobayashi N, Nishihashi T, Takahashi R, Nishikawa M, Takakura Y, Hashida M. Prevention of neutrophil-mediated hepatic ischemia/reperfusion injury by superoxide dismutase and catalase derivatives. J Pharmacol Exp Ther 2001;298:894-899.
16. Liu L, Zern MA, Lizarzburu ME, Nantz MH, Wu J. Poly(cationic lipid)-mediated in vivo gene delivery to mouse liver. Gene Ther 2003;10:180-187.
17. Wu J, Lizarzaburu ME, Kurth MJ, Liu L, Wege H, Zern MA, Nantz MH. Cationic lipid polymerization as a novel approach for constructing new DNA delivery agents. Bioconjugate Chem 2001;12;251-257.
18. Ozaki M, Deshpande SS, Angkeow P, Belian J, Lowenstein CJ, Dinauer MC, et al. Inhibition of the Rac1 GTPase protects against nonlethal ischemia/reperfusion-induced necrosis and apoptosis in vivo. FASEB J 2000;14:418-429.
19. Wege H, He HT, Chui MS, Liu L, Wu J, Giri R, et al. Telomerase reconstitution immortalizes human fetal hepatocytes without disrupting their differentiation potential. Gastroenterology 2003;124:432-444.
20. Shirahashi H, Wu J, Yamamoto N, Wege H, Wager B, Okita K, Zern MA. Differentiation of human and mouse embryonic stem cells along a hepatocyte lineage. Cell Transplant 2004;13:197-211.
21. Liu SL, Degli Esposti S, Yao T, Diehl AM, Zern MA. Vitamin E therapy of acute CCl_4-induced hepatic injury in mice is associated with inhibition of nuclear factor kappa B binding. Hepatology 1995;22:1474-1481.
22. Thompson C, Cloutier A, Bosse Y, Thivierge M, Le Gouill C, Larivee P, et al. CysLT1 receptor engagement induces AP-1- and NF-{kappa}B-dependent interleukin-8 expression. Am J Respir Cell Mol Biol 2006 (in press).
23. Batts KP, Ludwig J. Chronic hepatitis. An update on terminology and reporting. Am J Surg Pathol 1995;12:1409-1417.
24. Kupiec-Weglinski JW, Busuttil RW. Ischemia and reperfusion injury in liver transplantation. Transplant Proc 2005;37:1653–1656.
25. Malhi H, Gores GJ, Lemaster JJ. Apoptosis and necrosis in the liver: a tale of two deaths. Hepatology 2006;43:S31–S44.
26. Sonnenday CJ, Warren DS, Cooke SK, Dietz HC, Montgomery RA. A novel chimeric ribozyme vector produces potent inhibition on ICAM-1 expression on ischemic vascular endothelium. J Gene Med 2004;6:1394-1402.
27. McCarter SD, Badhwar A, Cott JR, Akyea TG, Bihari A, Dungey AA, et al. Remote liver injury is attenuated by adenovirus-mediated gene transfer of heme oyxgenase-1 during the systemic inflammatory response syndrome. Microcirculation 2004;11:587-595.
28. Pachori AS, Melo LG, Hart ML, Noiseux N, Zhang L, Morello F, et al. Hypoxia-regulated therapeutic gene as a preemptive treatment strategy against ischemia/reperfusion tissue injury. Proc Nat Acad Sci USA 2004;101:12282-12287.
29. Uehara K, Maruyama N, Huang CK, Nakano M. The first application of a chemiluminescence probe, 2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo[1,2-α]pyrazin-3-one (MCLA), for detecting O_2^- production, in vitro, from Kupffer cells stimulated by phorbol myristate acetate. FEBS Lett 1993;335:167-170.
30. Nakano M. Detection of active oxygen species in biological systems. Cell Mol Neurobiol 1998;18:565-579.
31. Midorikawa J, Maehara K, Yaoita H, Watanabe T, Ohtani H, Ushiroda S, Maruyama Y. Continuous observation of superoxide generation in an in situ ischemia-reperfusion rat lung model. Jpn Circ J 2001;65:207-212.
32. Guzik TJ, Channon KM. Measurement of vascular reactive oxygen species production by chemiluminescence. Methods Mol Med 2005;108:73-89.
33. Wheeler MD, Katuna M, Smutney OM, Froh M, Dikalova A, Mason RP, et al. Comparison of the effect of adenoviral delivery of three superoxide dismutase genes against hepatic ischemia-reperfusion injury. Human Gene Ther 2001;12:2167-2177.
34. Nguyen TH, Ferry N. Liver gene therapy: advances and hurdles. Gene Therapy 2004;11:S76–S84.
35. Dai L, Claxson A, Marklund SL, Feakins R, Yousaf N, Chernajovsky Y, Winyard PG. Amelioration of antigen-induced arthritis in rats by transfer of extracellular superoxide dismutase and catalase genes. Gene Therapy 2003;10:550-558.
36. Zwacka RM, Zhou W, Zhang Y, Darby CJ, Dudus L, Halldorson J, et al. Redox gene therapy for ischemia/reperfusion injury of the liver reduces AP1 and NF-kappaB activation. Nat Med 1998;4:698-704.
37. Zwacka RM, Zhang Y, Zhou W, Halldorson J, Engelhardt JF. Ischemia/reperfusion injury in the liver of BALB/c mice activates AP-1 and nuclear factor kappaB independently of IkappaB degradation. Hepatology 2000;32:278-288.
38. Ghavami S, Hashemi M, Kadkhoda K, Alavian SM, Bay GH, Los M. Apoptosis in liver diseases—detection and therapeutic applications. Med Sci Monit 2005;11:RA337-RA345.

AQ: 6

AQ: 7