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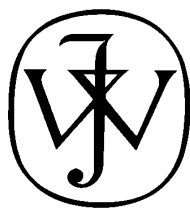
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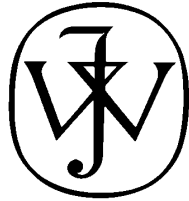
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Liposome-Mediated Extracellular Superoxide Dismutase Gene Delivery Protects Against Acute Liver Injury in Mice

AQ: 1

Jian Wu,[1] Li Liu,[1] Roy D. Yen,[1] Andreea Catana,[1] Michael H. Nantz,[2] and Mark A. Zern[1]

Our previous study demonstrated that polycationic liposomes are highly stable in the bloodstream and represent an effective agent for liver gene delivery. We report here that liposome-mediated extracellular superoxide dismutase (EC-SOD) gene delivery successfully prevented acute liver injury in mice. The therapeutic efficacy of EC-SOD gene delivery by polycationic liposomes was determined against the toxicity of superoxide anions and hydroxyethyl radicals in HepG2 cells and in a mouse model of acute liver injury caused by D-galactosamine and lipopolysaccharide intoxication. Transfection of HepG2 cells with an EC-SOD plasmid led to a striking increase in superoxide dismutase activity in the medium. The transfected cells had much less cell death after reactive oxygen species exposure compared with untransfected or control plasmid-transfected cells. In a model of acute liver injury, serum alanine aminotransferase levels in mice receiving portal vein injections of EC-SOD lipoplexes were much lower than in those receiving normal saline, liposomes alone, or control lipoplexes. Liver histology confirmed that there was less cell death in the EC-SOD lipoplex-treated group. Quantitative reverse transcriptase polymerase chain reaction showed a 55-fold increase in human EC-SOD gene expression in the liver of mice injected with EC-SOD lipoplexes. Serum superoxide dismutase activity in EC-SOD lipoplex-treated mice was higher than in the control groups; this was associated with higher liver glutathione levels and reduced lipid peroxidation. In conclusion, polycationic liposome-mediated EC-SOD gene delivery protects against reactive oxygen species toxicity *in vitro* and against lipopolysaccharide-induced acute liver injury in D-galactosamine–sensitized mice. (HEPATOLOGY 2004;40:000–000.)

Formation of reactive oxygen species (ROS) occurs in a variety of forms of liver injury and fibrogenesis. Common free radicals, such as superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals ($HO^{\bullet-}$), hydrogen peroxide (H_2O_2) or hydroxyethyl radicals (HERs), are generated during drug toxicity, ischemia/reperfusion, and alcohol metabolism, in addition to reactive intermediate metabolites of hepatotoxins or drugs.[1] These ROS are responsible for necrosis and/or apoptosis of hepatocytes and sinusoidal endothelial cells, the activation of Kupffer cells (thus causing the second phase of liver inflammation), and the activation of hepatic stellate cells and subsequent hepatic fibrogenesis; therefore, antioxidative treatment appears to be an effective means of attenuating liver injury and fibrosis in liver diseases.[2] Our previous studies have shown that using antioxidants such as vitamins E and C or free radical scavengers such as catalase or superoxide dismutase (SOD) prevented carbon tetrachloride, D-galactosamine (GalN), or bromobenzene-induced acute hepatocellular damage.[3–5] Therefore, our speculation is that treatment with antioxidants or free radical scavengers

will prevent or attenuate acute liver injury caused by hepatotoxins, hepatotoxic drugs, Fe^{2+} or Cu^{2+} overload, alcohol abuse, or ischemia/reperfusion.[6]

There are three isozymes of SOD. The copper–zinc-containing form of 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 a manganese-containing enzyme localized in the matrix of mitochondria. The third type is extracellular SOD (EC-SOD), which is a secretory glycoprotein and is composed of four 30-kd subunits, each containing a Cu and a Zn atom.[7] EC-SOD is localized primarily in the interstitial matrix of tissue and is characterized by its high affinity for heparan sulfate binding.[8] The importance of EC-SOD in normal liver and pathological conditions has not been fully elucidated. It may play a role in regulating O_2^- levels in the extracellular space, because O_2^- poorly penetrates the cell membrane when it can be cleared by intracellular copper–zinc SOD.[7,9] Thus EC-SOD may be an important factor in the extracellular space to degrade O_2^- generated during pathological processes and to protect tissues from ROS toxicity.

ROS that exist in the extracellular space appear to mediate the interactions among different cell types. Therefore, treatments that reduce the production of ROS, inhibit their release, or inactivate their toxic action should attenuate ROS-associated liver injury. Emerging strategies include the administration of antioxidants, SOD enzymes, or mimics[10–12] and the gene delivery of free radical scavengers, such as copper–zinc SOD via adenoviral vectors.[13] Adenoviral EC-SOD vectors were also used to protect rabbits from myocardial infarction damage[14] and from ischemia/reperfusion-associated liver injury in mice.[15] Gene delivery will overcome the short half-life of the enzyme in the body. However, obvious drawbacks exist with adenoviral vectors, such as liver toxicity, immunogenicity of viral products, and so forth.

Many formulations of liposomes as gene transfer agents are used for *in vitro* gene transfection, and their *in vivo* applications were considered to be limited due to their interaction with plasma proteins, transient gene transfer, and relative lower levels of transgene expression compared with some viral vectors.[16–17] With modifications in lipid structure, formulations, and targeting approaches, significant improvements in gene transfer efficacy have been achieved.[18–22] A series of successful attempts in genetic correction,[19,22] cancer gene therapy,[23] and targeting liver gene delivery[24] have been reported. In an attempt to establish a more effective liposomal formulation, we have developed a polycationic lipid (PCL) and formulated polycationic liposomes with cholesterol (Chol).[25] The PCL-Chol formulation we developed is nontoxic, binds the least to plasma proteins, and displays high gene transfer efficacy to the liver when compared with the commonly used 1,2-bis(dioleoyloxy)-3-(trimethylamonio)propane–Chol or 1,2-bis(dioleoyloxy)-3-(trimethyla-monio)propane–L-α dioleoyl phosphatidylethanolamine formulations.[26] We also developed a noninvasive approach to promote hepatocyte proliferation by subcutaneously administering thyroid hormone, which led to profound reporter gene expression in mouse liver.[26] In this study we report that our PCL-Chol liposome-mediated EC-SOD gene delivery protected against superoxide anion- or hydroxyethyl radical–induced HepG2 cell death as well as lipopolysaccharide-induced acute liver injury in GalN-sensitized mice.

Materials and Methods

Subcloning EC-SOD Expression Plasmid. Plasmid pUC18-ECSOD (kindly provided by Dr. Stefan Marklund, Department of Medical Biosciences, Umeå University, Umeå, Sweden[27]) was digested by EcoRI, and the EC-SOD gene was ligated into pEGFP-C1 or pIRES2-EGFP from Clontech Laboratories, Inc. (Palo Alto, CA) at the multiple cloning site to form new plasmids, pEGFP-C1-ECSOD and pIRES2-EGFP-ECSOD. The orientation of the ligated fragment of the EC-SOD gene in the plasmids was verified by restriction enzymes (BamHI) and sequencing, using a specific forward primer. The resulting plasmid DNA was transformed into competent *Escherichia coli* cells from Gibco Life Science Technologies (Grand Island, NY). The plasmid DNA was extracted from overnight cultures of the competent cells and purified by affinity chromatography with an Endofree plasmid extraction kit from Qiagen, Inc. (Valencia, CA). The quality of the DNA was determined by UV spectroscopy and agarose gel electrophoresis after cleavage by specific restriction endonucleases. The DNA concentration was quantitated spectrophotometrically. The plasmid DNA was frozen at −20°C and diluted to 1 μg/μL in water for *in vitro* transfection, or in normal saline for *in vivo* gene delivery, prior to use.[25]

Transfection of EC-SOD Plasmids in HepG2 Cells and Measurement of SOD Activity in Culture Medium. HepG2 cells were cultured in minimum essential medium plus 10% fetal bovine serum and antibiotics and were transiently transfected with either the control plasmid (pEGFP-C1) or EC-SOD plasmid (pEGFP-C1-EC-SOD) using Fugene 6 (Roche Molecular Biochemicals, Indianapolis, IN), when they were 50%–70% confluent. One day after the transfection, the culture medium was changed to serum-free medium. Culture medium was collected 48 hours after the medium change for the spectro-

Table 1. Design of Animal Experiment

Group	T₃ (4 mg/kg Subcutaneously)	Treatments (Portal Vein Injection of Lipoplexes)	Ga1N/LPS (500 mg + 25 μg/kg Intraperitoneally)
Saline	+	Saline (300 μL)	+
Liposome	+	PCL-Chol (200 μL)	+
Liposome + control Plasmid DNA	+	PCL-Chol + pEGFP-C1 (100 μg DNA)	+
Liposome + EC-SOD Plasmid DNA	+	PCL-Chol + pEGFP-C1-EC-SOD (100 μg DNA)	+

Time line of the experiment

T₃		Lipoplexes	Ga1N/LPS	Sacrifice
↓		↓	↓	↓

| Day 0 | 1 | 2 | 3 | 4 |
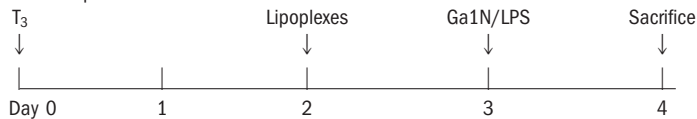
photometric determination of SOD activity with a commercially available kit from Calbiochem, Inc. (San Diego, CA). For stable transfection, Hep3B cells were cultured with minimum essential medium plus 10% fetal bovine serum and antibodies, and were transfected with either pEGFP-C1-ECSOD or control plasmids by a PCL-Chol formulation as we reported previously.[25] The transfected cells were subjected to geneticin (G418) selection (350 μg/mL medium) over 1 week. G418-resistant cells were grown in the medium containing G418 until it was changed for serum-free medium. One to three days after the cells were cultured in the serum-free medium, SOD activity in cell culture medium and cell lysates was determined by the kit mentioned above.

Necrosis and/or Apoptosis in HepG2 Cells Induced by Superoxide Anions or HERs. One day after HepG2 cells were transiently transfected with either the control plasmid or EC-SOD plasmid, the cells were subjected to pretreatment with the glutathione-depleting agent buthionine sulfoximine (BSO, 0.3 mM) for 18 hours and a subsequent exposure to the superoxide anion–generating system hypoxanthine (HX, 1 mM) and xanthine oxidase (XO, 2 mU) for 4–7 hours.[28] In separate experiments, the transfected cells were subsequently exposed to a HER-generating system, which consists of H_2O_2 (0.1 mM), ferrous ammonium sulfate (20 μM), and ethanol (200 mM) for 8 hours.[29]

After the cells were exposed to either $O_2^{\bullet-}$ or HER, culture medium was collected for the determination of lactate dehydrogenase leakage (Roche Molecular Biochemicals, Indianapolis, IN), which is employed as an indicator of cell necrosis.[25] The percentage of cell death was calculated according to a formula provided by the manufacturer. After the ROS exposure, the cells cultured on LabTech chamber slides (Fisher Scientific, Inc., Santa Clara, CA) were fixed with 1% paraformaldehyde and stained with an *in situ* apoptosis detecting kit, Apoptag

(Intergen Co., Purchase, NY), which uses rhodamine-conjugated antidigoxigenin for final images.

Liposome Generation and Size Measurements. PCL was synthesized and validated as previously described[25] and the PCL-Chol liposome formulation was generated in a molar ratio of 3:1 as described in detail previously.[26] The liposome size was measured after sonication by a laser-based, submicron particle size analyzer from Beckman Coulter, Inc., as described previously.[5,25] The size of liposomes at different times of generation is between 200–250 nm before use. For animal experiments, PCL-Chol liposomes were complexed with plasmid DNA at a charge ratio of 5:1.[26] For each mouse, 200 μL of liposome suspension containing 0.3 μmol PCL and 0.1 μmol cholesterol was injected via the portal vein.

GalN/Lipopolysaccharide-Induced Acute Liver Injury and Its Prevention by Polycationic Liposome-Mediated EC-SOD Gene Delivery. Polycationic liposome-mediated EC-SOD gene delivery to the liver was conducted according to our previous description.[26] The animal experimental protocol was approved by the Animal Care and Use Administrative Advisory Committee of the University of California–Davis, according to guidelines of the National Institutes of Health. C57BL/6 mice (Charles River Laboratory, Wilmington, MA) were fed a pellet diet and water *ad libitum* and kept on a 12 hour-light/dark cycle. Animals were randomly divided into 4 groups, and all received 1 injection of thyroid hormone (triiodothyronine [T₃], 4 mg/kg subcutaneously) to stimulate hepatocyte proliferation. Two days after T₃ injection, the animals were treated following a protocol outlined in Table 1. Animals were anesthetized with pentobarbital for the operation (60 mg/kg intraperitoneally). Polycationic liposomes alone (200 μL), or complexes with plasmid DNA (lipoplexes) were injected via the portal vein (100 μg control plasmid pEGFP-C1 or

pEGFP-C1-ECSOD per mouse). One day after the lipoplex injection, animals were exposed to GalN (500 mg/kg intraperitoneally) plus lipopolysaccharide (LPS, 25 μg/kg intraperitoneally). The animals were sacrificed 24 hours after the GalN/LPS intoxication, and the blood was collected for measurement of alanine aminotransferase (ALT) levels (Sigma Chemical Co., St. Louis, MO) and serum SOD activity as described previously. Part of the liver tissue was fixed in 10% buffered formalin for routine paraffin embedding and hematoxylin-eosin staining. Some liver tissue was snap-frozen for sectioning. The frozen sections were subsequently fixed in 10% buffered formalin and examined for green fluorescence protein (GFP) expression under a fluorescent microscope. The images were recorded with a digital videocamera.[25] The reduced form of glutathione (GSH), malondialdehyde (MDA) and 4-hydroxyalkenal (HAE) levels in the liver tissue were determined spectrophotometrically by commercially available kits (OXISResearch, Portland, OR) according to the manufacture's instructions and expressed as nmol/mg protein in tissue.

Determination of Human EC-SOD Gene Expression in Mouse Liver Tissue via Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction. Messenger RNA levels of human EC-SOD in mouse liver tissue were determined by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) using mouse β-actin as a housekeeping gene control. RNA was extracted from the mouse liver tissue of 3 independent experiments using TRIzol (Invitrogen, Carlsbad, CA) and quantitated via absorbance of a 260-nm wavelength. Complementary DNA was generated via reverse transcription of DNase I–digested RNA employing ThermoScript RT-PCR Systems (Invitrogen). Amplification was performed with Platinum PCR Super Mix (Invitrogen) and a primer pair of the human EC-SOD gene in ABI Prism 7700 Thermal Cycler (Applied BioSystems, Foster City, CA).[30] The sequences of the primer pair are: forward primer, 5'-AACTGCCCC-GCGTCTTC-3'; reverse primer, 5'-GCCAAACATTC-CCCCAAAG-3'; and fluorescent probe, 6-carboxyfluorescein-5'-TGTTTCGCATCCACCGCCACC-3'. The concentrations for forward and reverse primers were 900 nM and 50 nM, respectively, both of which were optimized for an annealing temperature of 60°C. Following 40 cycles of amplification in the above condition, semi–log amplification curves were evaluated using comparative quantification ($\Delta\Delta C_T$).[30] Expression levels were normalized to mouse β-actin housekeeping gene control. The relative gene expression in different groups was calculated based on the average level of the saline control group.

Statistical Analysis. Most data were expressed as mean ± SEM and evaluated using ANOVA and Newman-

Keuls test for multiple comparisons among groups. Student's unpaired *t* test was used for the comparison between two groups. Wilcoxon Signed Rank test was employed for evaluating quantitative RT-PCR data, followed by q tests for multiple comparisons among groups. A *P* value less than .05 was considered statistically significant.

Chemicals and Reagents. Dulbecco's Modified Eagle Medium, cell culture supplements, and fetal bovine serum were purchased from Invitrogen, Inc. (Gaithersburg, MD). GalN, LPS, HX, and XO were the products of Sigma Chemical Co. Most other chemicals used were commercially available reagents of analytical grade.

Results

SOD Activity in Culture Medium After Transient or Stable Transfection of an EC-SOD Plasmid in Hepatoma Cells. After verification with restriction enzyme cleavage and sequencing, the new subcloned EC-SOD plasmid, pEGFP-C1-ECSOD, was used to transfect human hepatoma cell lines HepG2 and Hep3B, either transiently or stably. After transient transfection in HepG2 cells with pEGFP-C1-ECSOD plasmid DNA, SOD levels in the culture medium were elevated from the background to 28 units/mL at 72 hours (Fig. 1A). Untransfected cells or cells transfected with the control plasmid, pEGFP-C1, did not show any increase in SOD activity in medium. Hep3B cells were transfected with either the control plasmid or EC-SOD plasmid using our PCL-Chol liposome formulation, and the transfected cells were selected by the addition of G418. SOD activity in culture medium and cell lysates from EC-SOD plasmid-transfected Hep3B cells was markedly increased 24 and 72 hours after the medium change compared with the control plasmid-transfected cells ($P < .01$) (Fig. 1B). The SOD activity measured reflects total activity of all three SOD isoforms in the cell lysates.

Protection Against Toxicity of Superoxide Anions and HERs by EC-SOD Overexpression in HepG2 Cells. One day after the transfection of HepG2 cells with either control plasmid or EC-SOD plasmid, the cells were first exposed to BSO for 18 hours, and subsequently to HX and OX for 7 hours. As shown in Fig. 2A, lactate dehydrogenase leakage from the cells transfected with pEGFP-C1-ECSOD at a late time point (7 hours after exposure to HX/OX) was significantly lower than untransfected cells or cells transfected with the control plasmid, pEGFP-C1 ($P < .01$). EC-SOD plasmid transfection also markedly diminished the number of Apoptag-positive cells after HX/XO exposure (Fig. 2B).

In separate experiments, after HepG2 cells were treated with BSO, they were subsequently exposed to a HER-
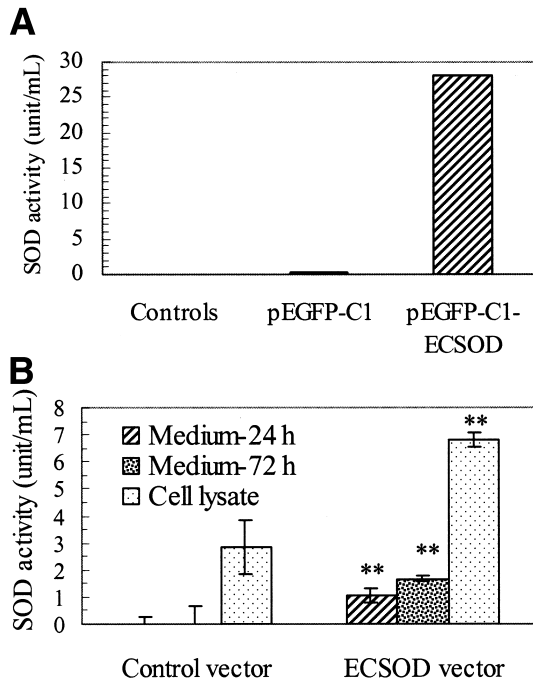
Fig. 1. SOD activity in EC-SOD plasmid-transfected HepG2 and Hep3B cells. (A) HepG2 cells were transfected with either the control plasmid (pEFGP-C1) or EC-SOD plasmid (pEGFP-C1-ECSOD) using Fugene 6 (Roche Molecular Biochemicals). One day after the transfection, culture medium was changed to serum-free medium, and SOD activity in medium was measured 72 hours after the transfection. (B) Hep3B cells were transfected with either pEGFP-C1 or pEGFP-C1-ECSOD by our polycationic liposomes (PCL-Chol) as described in Materials and Methods. Two to three days after the transfection, the cells were subjected to G418 selection. G418-resistant cells were expanded and SOD activity in culture medium and cell lysates was determined spectrophotometrically 1–3 days after culture medium was changed to serum-free medium. The data are summarized from three experiments (**$P < .01$ compared with control plasmid-transfected Hep3B cells). Abbreviations: SOD, superoxide dismutase; EC-SOD, extracellular superoxide dismutase.

generating system for 8 hours. It is clear that cells transfected with the EC-SOD plasmid had a much lower percentage of Apoptag-positive cells (Fig. 3E) compared with untransfected controls (Fig. 3A) or to the control plasmid-transfected cells (Fig. 3C). Apoptag-positive cell counts shown in Fig. 3G quantitate the findings.

PCL-Chol–Mediated EC-SOD Gene Delivery to Mouse Liver and Protection Against GalN/LPS-Induced Acute Liver Toxicity. In a separate pilot experiment, subcutaneous T_3 injection did not change serum ALT levels (<20 units/mL, n = 3) in normal mice. Injection of PCL-Chol liposomes via either the tail vein or portal vein caused a slight serum ALT increase (25 ± 2, 35 ± 3 units/mL, n = 3) in normal mice 48 hours after the injection. Injection of the control lipoplexes (PCL-Chol-pEGFP-C1 complexes) via the tail vein or portal vein caused a similar increase in serum ALT levels (30 ± 0, 29 ± 1 units/mL, n = 3). Although these values are

statistically higher than untreated controls (16 ± 3 units/mL, n = 3, $P < .01$), the data indicate that neither PCL-Chol liposomes nor the control lipoplexes are significantly toxic to the liver. Following a protocol described in Table 1 and the time line, we first injected T_3. Two days after T_3 injection, PCL-Chol liposome or lipoplexes were injected via the portal vein. One day after liposome or lipoplex injection, all animals were challenged intraperitoneally with GalN plus LPS, then sacrificed one day later. Serum ALT levels in animals receiving liposomes alone or lipoplexes with control plasmid were similar to levels in saline controls ($P > .05$). Serum ALT
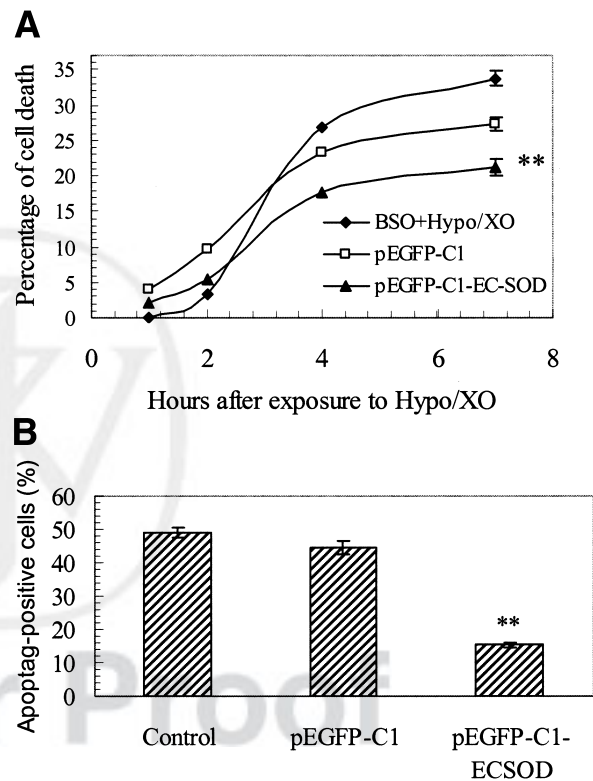

Fig. 2. Protection against superoxide-induced cell death by EC-SOD plasmid transfection. (A) HepG2 cells were transfected with either the control plasmid (pEGFP-C1) or EC-SOD plasmid (pEGFP-C1-ECSOD) by Fugene 6 (Roche Molecular Biochemicals). One day after the transfection, the cells were first treated with BSO for 18 hours, then subsequently exposed to hypoxanthine (HX, 1 mM) and xanthine oxidase (XO, 2 mU) for 4–7 hours. Lactate dehydrogenase leakage was measured to assess necrosis. The data are a mean of triplicates of 1 representative experiment (**$P < .01$ compared with untransfected controls or cells transfected with pEGFP-C1). (B) HepG2 cells were transfested with either control plasmid or EC-SOD plasmid and were exposed to BSO plus HX/XO. An *in situ* apoptosis detection kit (Apoptag, Intergen Co.) was employed to stain apoptotic (Apoptag-positive) cells in chamber slides after fixation. Apoptag-positive cells in 10 random fields were counted and expressed as a percentage of total cells. The data are summarized from 3 independent experiments. **$P < .01$ compared with untransfected controls or pEGFP-C1–transfected cells. Abbreviation: BSO, buthionine sulfoximine; Hypo, hypoxanthine; XO, xanthine oxidase; EC-SOD, extracellular superoxide dismutase.
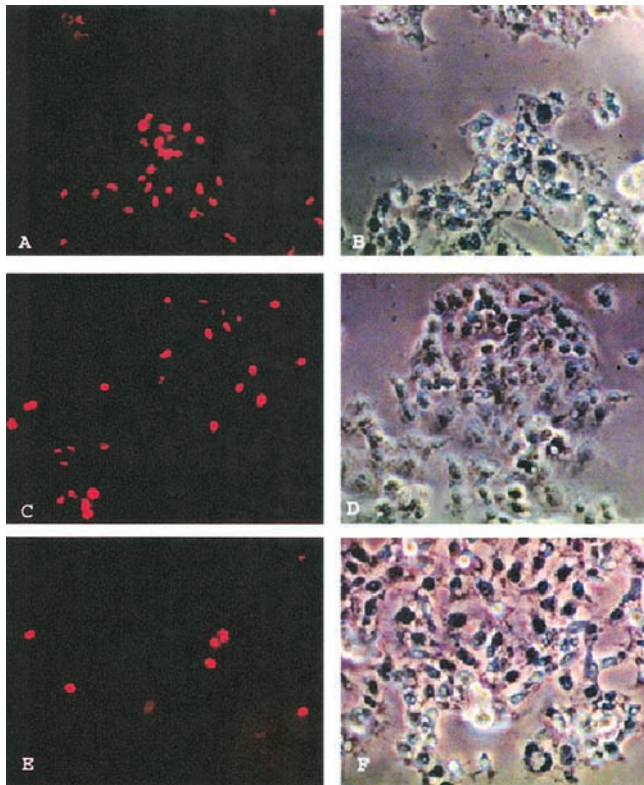
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Fig. 3.   Representative images of Apoptag-positive cells after exposure to HERs. An *in situ* apoptosis detection kit was employed to stain apoptotic (Apoptag-positive) cells in chamber slides after fixation. The experimental protocol is the same as in Fig. 2B. (A, C, E) Apoptag-positive cells shown in red by staining with an *in situ* apoptosis detection kit (Apoptag, Intergen Co. (B, D, F) plain images of the same field. (A, B) untransfected images; (C, D) control plasmid-transfected images; (E, F) EC-SOD plasmid-transfected images. (G) Apoptag-positive cells in 10 random fields were counted and expressed as a percentage of total cells. **$P < .01$ compared with untransfected controls or pEGFP-C1–transfected cells.

levels in animals receiving EC-SOD lipoplexes (PCL-Chol-pEGFP-C1-ECSOD) were markedly lower than the other three groups ($P < .01$) (Fig. 4). Liver histology revealed a similar degree of massive cell death and inflammatory infiltration in GalN/LPS-intoxicated animals plus portal vein injection of saline, liposomes (PCL-Chol) or control lipoplexes (PCL-Chol-pEGFP-C1) (Fig. 5A–5C). Much less cell death was found in the liver of animals receiving EC-SOD lipoplexes (Fig. 5D) before the GalN/LPS challenge, compared with the other three groups. Therefore, the liver histology findings are consistent with serum ALT level alterations, and liver injury in mice receiving EC-SOD lipoplexes was markedly attenuated compared with those treated with saline control, PCL-Chol liposomes only, or control lipoplexes.

Liver frozen sections were examined for GFP expression. Intensive GFP expression was found in the liver sections from animals receiving control lipoplexes or EC-

SOD lipoplexes (Fig. 6C and 6D), but not in the sections from saline control mice or from liposome-injected mice (Fig. 6A and 6B).

Human EC-SOD gene expression in the livers of mice receiving injections of either PCL-Chol liposomes or control lipoplexes did not significantly differ from the saline control group, as evaluated by real-time quantitative RT-PCR. Mouse EC-SOD does not cross-hybridize with the human gene. The liver human EC-SOD gene expression levels in mice receiving EC-SOD lipoplex injection were approximately 55-fold higher than the other 3 groups (Fig. 7A). Serum SOD activity was measured in all GalN/LPS-intoxicated mice, and the data showed that the total SOD activity in the serum of animals receiving portal vein injection of EC-SOD lipoplexes was higher than in those receiving the injection of liposomes alone or control lipoplexes ($P < .01$) (Fig. 7B). The SOD activity measured reflects the 3 human and mouse isoforms of SOD in serum. These results indicate that the gene was successfully delivered to the mouse liver with our polycationic liposomes and that the gene was highly expressed in the mouse liver.

GSH Preservation and Reduced Lipid Peroxidation by EC-SOD Gene Delivery in GalN/LPS-Intoxicated Mice. As shown in Fig. 8, LPS challenge in GalN-sensitized mice led to enhanced lipid peroxidation as indicated by MDA/HAE levels and decreased levels of the reduced form of glutathione in the liver ($P < 0.01$) compared with untreated controls. Animals receiving EC-SOD gene delivery had preserved GSH levels ($P < .05$) and reduced MDA/HAE levels ($P < .05$) in their livers in comparison with liposome controls or control plasmid-transfected animals (Fig. 8A and 8B). Both liver GSH and
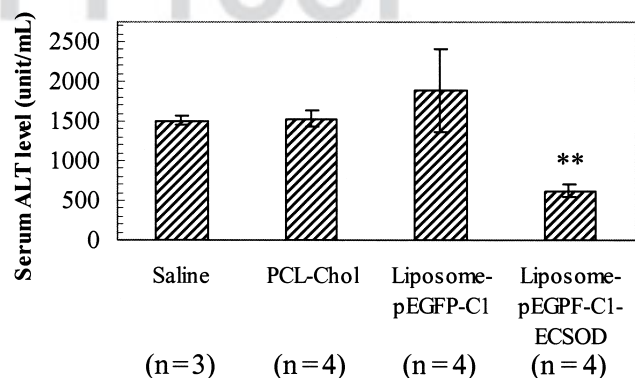

Fig. 4.   Serum ALT levels in mice exposed to GalN/LPS with or without PCL-Chol–mediated EC-SOD gene delivery. The experimental protocol was described in detail in Materials and Methods. Data are summarized from 3 independent experiments. **$P < .01$ compared with saline controls, liposomes (PCL-Chol) alone, or pEGFP-C1–transfected cells. Abbreviations: ALT, alanine aminotransferase; PCL, polycationic lipid; Chol, cholesterol.
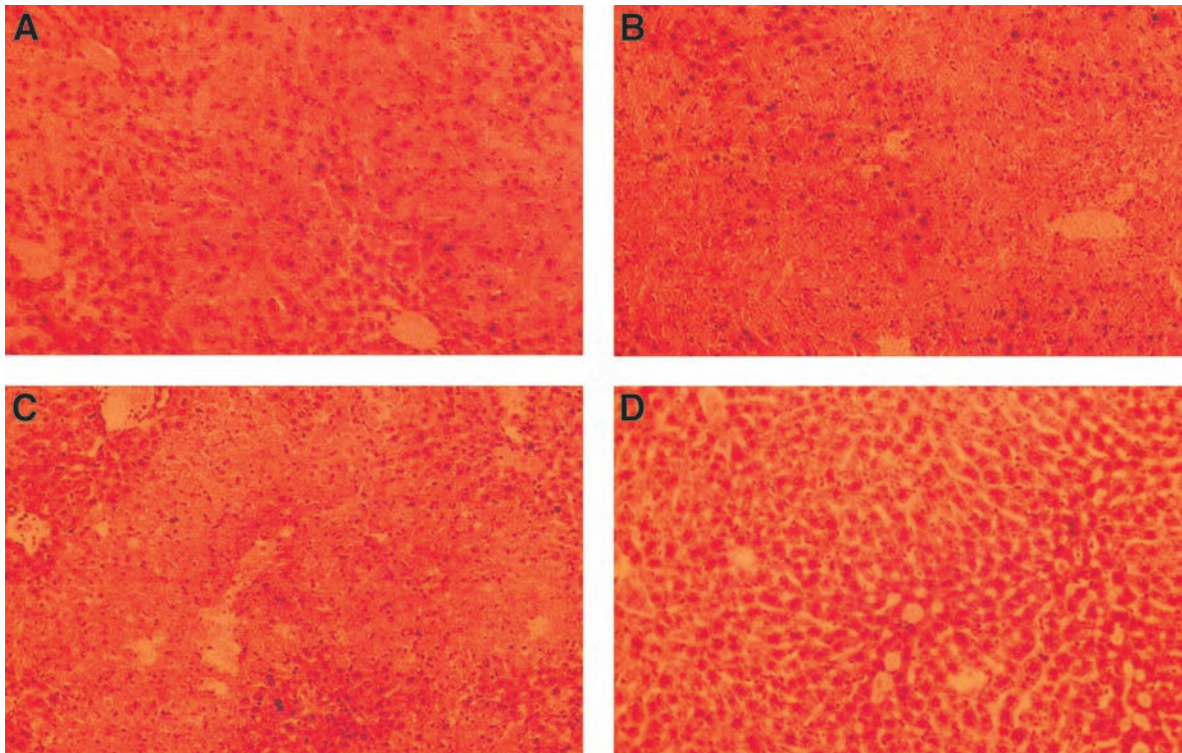
Fig. 5.   Representative micrographs of liver histology. Liver sections were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin-eosin. (A) GalN/LPS treatment with saline control. (B) GalN/LPS treatment with liposomes (PCL-Chol) alone. (C) GalN/LPS treatment plus PCL-Chol–mediated control plasmid (pEGFP-C1) transfer. (D) GalN/LPS treatment plus PCL-Chol–mediated EC-SOD gene (pEGFP-C1-ECSOD) transfer (original magnification $\times$100).

MDA/HAE levels in animals receiving EC-SOD gene delivery were close to those in untreated controls without GalN/LPS intoxication ($P > 0.05$).

Discussion

In the present study, we first tested whether the newly subcloned EC-SOD plasmid works in hepatoma cell lines after transfection. Elevated SOD activity was seen in culture medium from HepG2 cells transiently transfected and from Hep3B cells stably transfected with the new recombinant EC-SOD plasmid, pEGFP-C1-ECSOD. Increased SOD levels in culture medium demonstrate the characteristic extracellular location of the enzyme. We then treated control plasmid-transfected or EC-SOD plasmid-transfected HepG2 cells initially with BSO, a GSH-depleting agent,[31] and subsequently with either $O_2^{\bullet}$ generated from HX and XO[32] or HER using a HER-generating system.[29] The cells transfected with pEGFP-C1-ECSOD showed less lactate dehydrogenase leakage and a lower percentage of Apoptag-positive cells compared with those transfected with the control plasmid or untransfected cells. The results indicate that the overexpression of EC-SOD makes cells more resistant to $O_2^{\bullet}$ or other $O_2^{\bullet}$-derived ROS, such as HO$^{\bullet}$ or per-

oxynitrite anions (ONOO$^{\bullet}$).[1,33] A similar finding was achieved in HepG2 cells when they were treated with HER, which occurs in ethanol metabolism and probably contributes to the pathogenesis of alcohol-associated liver injury.[34]

In our previous studies,[25–26] we reported that our polycationic liposomes are serum-resistant *in vitro* and interact the least with plasma proteins in the bloodstream when compared with two other commonly used cationic liposome formulations.[18,35] In the present study, we employed the same approach as reported in our previous study[26] to deliver the EC-SOD gene with our polycationic liposome formulation to the liver. The markedly enhanced human EC-SOD gene expression in the mouse liver tissue and elevated serum SOD activity in the animals receiving EC-SOD lipoplex injection via the portal vein confirmed that the hepatocytes were transfected, expressed the functional gene, and released the active enzyme into the bloodstream. Moreover, due to the binding properties to glycosaminoglycans in the extracellular space, the measurement of SOD or EC-SOD activity may underestimate the actual EC-SOD activity in the bloodstream.[36] Finally, both the control plasmid- and EC-SOD plasmid-transfected liver cells showed positive GFP ex-

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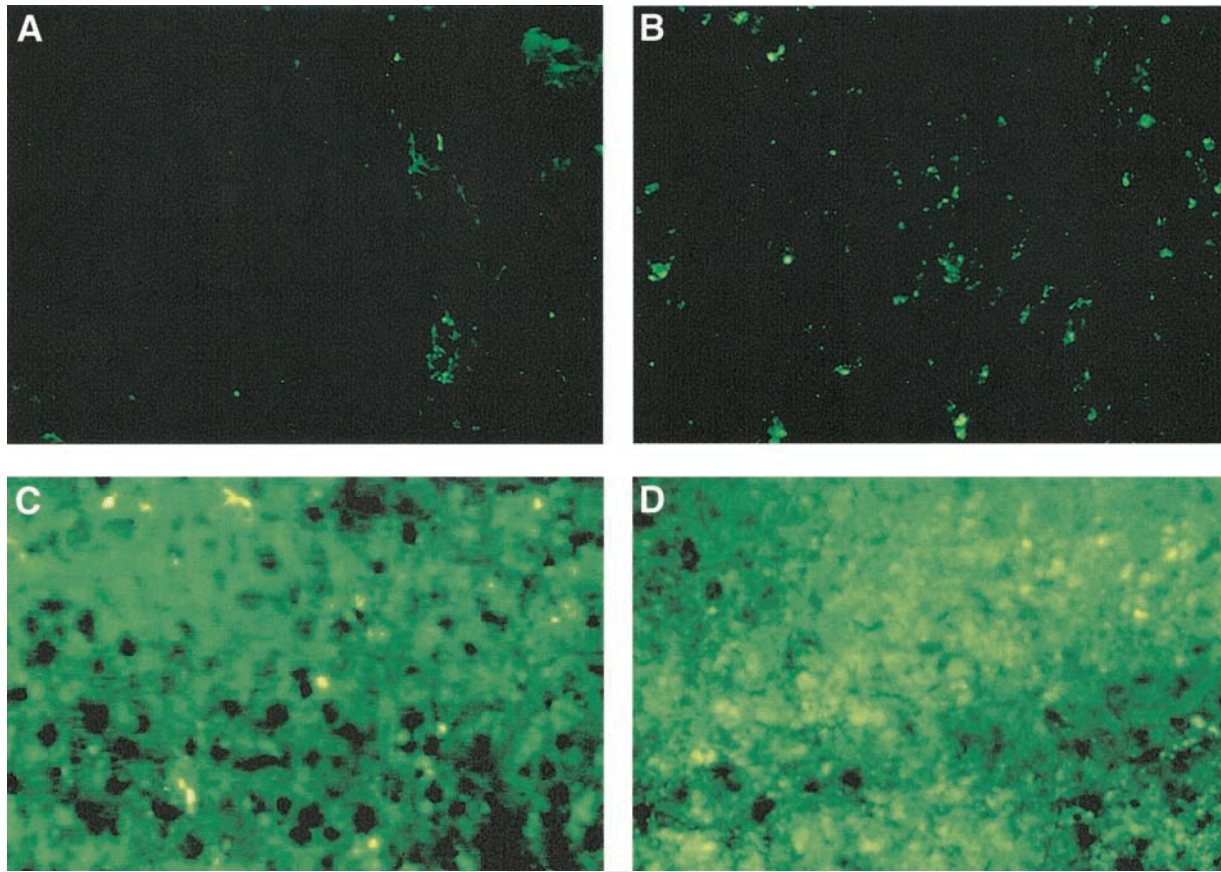
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Fig. 6. Representative GFP images in the liver of mice receiving PCL-Chol–mediated GFP plasmid transfer. Frozen liver tissues were collected when animals were sacrificed (2 days after liposome-mediated gene delivery via portal vein injection), sectioned with a Crystat, and fixed in 10% buffered formalin. GFP images were recorded in a fluorescent microscope with a digital camera (original magnification ×100). (A) GalN/LPS treatment with saline control. (B) GalN/LPS treatment with liposomes (PCL-Chol) alone. (C) GalN/LPS treatment plus PCL-Chol–mediated control plasmid (pEGFP-C1) transfer. (D) GalN/LPS treatment plus PCL-Chol–mediated EC-SOD gene (pEGFP-C1-ECSOD) transfer.

pression at a high rate (see Fig. 6), which further confirmed the effectiveness of our polycationic liposome-mediated gene transfer to the liver.[26]

LPS-induced acute liver injury in GalN-sensitized mice is a common model of ROS-associated toxicity, in which enhanced lipid peroxidation is thought to be responsible for the hepatocellular death, via necrosis or apoptosis.[37] Elevated levels of tumor necrosis factor α and ROS contribute to the enhanced lipid peroxidation and GSH depletion.[38–40] Findings in this study, including elevated serum ALT levels, massive cell death, and inflammatory infiltration in the liver sections, as well as decreased GSH levels and elevated MDA/HAE in the liver tissue, demonstrate the feasibility of the model as a means of determining the therapeutic efficacy of liposome-mediated EC-SOD gene delivery. Consequently, a marked decrease in serum ALT levels, improved liver histology, preserved GSH content, and decreased MDA/HAE content in the liver tissue from animals receiving the portal vein injection of EC-SOD lipoplexes compared with those receiving saline, liposomes alone, or control lipo-

plexes documented the efficacy of the EC-SOD gene delivery. The protection may be attributed to the enhanced scavenging activity of EC-SOD in the liver cells and in the interstitial space. Our results are consistent with a recent report that topical transfer of EC-SOD gene ameliorated antigen-induced arthritis in rats[40] and that adenoviral vector-mediated EC-SOD gene delivery attenuated acetaminophen liver toxicity.[41] Slightly increased ALT levels were seen in animals receiving an injection of polycationic liposomes or control lipoplexes without GalN/LPS intoxication. This slight serum ALT increase indicates that neither PCL-Chol liposomes nor the lipoplexes are significantly toxic. Slightly increased—but not statistically significant—serum ALT levels in the PCL-Chol-pEFGP-C1 group suggests a possible toxic effect of plamsid DNA due to the "CpG" motif from the bacterial genomic sequence during the plasmid transformation.[42] This effect can be eliminated by further purification of plasmid DNA.[43] Our results suggest that liposome-mediated delivery of a functional gene may be at least as good as adenoviral delivery,[41] and perhaps better. This is because high titers
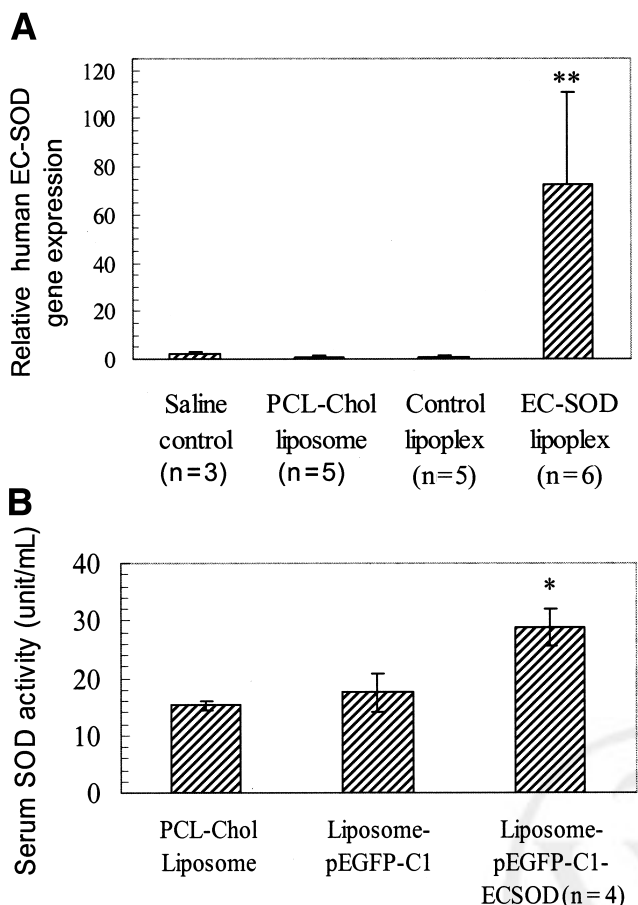
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Fig. 7. Human EC-SOD gene expression in mouse liver and serum SOD activity in mice receiving liposome-mediated EC-SOD gene delivery. (A) Human EC-SOD messenger RNA levels in mouse liver tissue were determined with real-time quantitative RT-PCR using mouse β-actin as a housekeeping gene control. The relative human EC-SOD gene expression levels in three other groups were calculated based on an average level in the control group. (B) Serum SOD activity after PCL-Chol–mediated EC-SOD gene delivery and subsequent GalN/LPS toxicity. Serum total SOD activity was measured spectrophotometrically 2 days after portal vein injection of liposomes or lipoplexes and subsequent GalN/LPS toxicity. *$P < .05$, **$P < .01$ compared with the 3 other groups. Abbreviations: EC-SOD, extracellular superoxide dismutase; PCL, polycationic lipid; Chol, cholesterol; SOD, superoxide dismutase.

ation through the injection of thyroid hormone before administering the lipoplexes. This approach is not toxic and resulted in a level of hepatocyte proliferation similar to partial hepatectomy,[26] and a high level of EC-SOD gene expression was detected in the mouse liver, leading to a functional consequence in the present study: protection against oxidative injury.

In conclusion, the findings in the present study demonstrate that the overexpression of extracellular SOD protects against either superoxide anion– or HER-induced
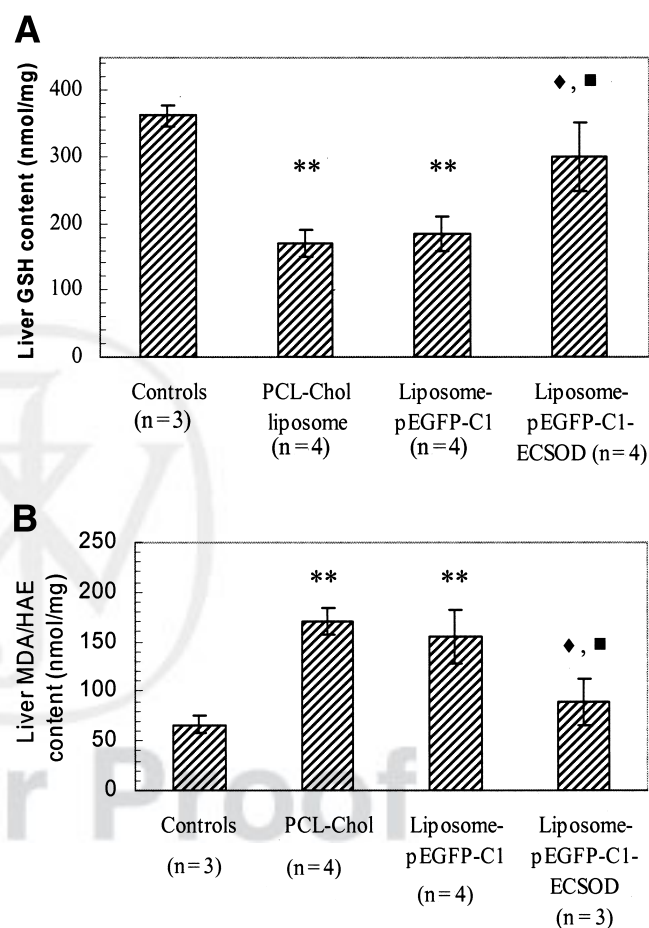
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Fig. 8. Liver GSH content and lipid peroxidation after EC-SOD gene overexpression and GalN/LPS toxicity. (A) GSH content in normal control liver and in the liver after portal vein injection of liposomes or lipoplexes and subsequent GalN/LPS toxic challenge. Liver GSH content was determined spectrophotometrically and expressed as nmol/mg protein of the tissue. The data are summarized from 3 experiments. ♦$P > .05$, **$P < .01$ compared with controls. ■$P < .5$ compared with either PCL-Chol liposomes or the liposome–pEGFP-C1 group. (B) MDA and HAE in normal controls or in the mouse liver after liposome-mediated EC-SOD gene delivery and subsequent GalN/LPS toxic challenge. Liver MDA and HAE levels were measured spectrophotometrically and expressed as nmol/mg protein of the tissue. Data are summarized from 3 independent experiments. ♦$P > .05$, ** $P < .01$ compared with controls. ■$P < 0.5$ compared to either PCL-Chol liposomes or the liposome–pEGFP-C1 group. Abbreviations: GSH, glutathione; PCL, polycationic lipid; Chol, cholesterol; MDA, malondialdehyde; HAE, 4-hydroxyalkenal. ABI Prism 7700 Thermal Cycler.

of adenoviruses (*i.e.*, titers high enough to be therapeutically significant) may cause liver injury through a direct toxic effect or through immunomediated damage.[44]

The fact that liposomes are nonimmunogenic enhances their role as a nonviral vector for gene delivery. When targeting approaches are desirable, liposomes can be employed to selectively deliver therapeutic genes to a preferential organ or cell type.[21,22,24] One disadvantage associated with nonviral gene delivery approaches and retroviral vectors is that a high level of transgene expression can be achieved only when targeted cells are in a proliferative state.[26] We employed a noninvasive approach to promote hepatocyte prolifer-

necrosis/apoptosis in HepG2 cells, and that polycationic liposome-mediated EC-SOD gene delivery markedly attenuated GalN/LPS-induced acute liver injury in mice. To our knowledge, this is a first report, which demonstrates that polycationic liposome-mediated EC-SOD gene delivery to the liver represents a potential therapy for ROS-associated liver injury.

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AQ5: Please provide reference citation for previously described methods.

AQ6: Please verify/provide manufacturer name and location for TRIzol, ThermoScript RT-PCR Systems,Platinum PCR Super Mix, and

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