Effects of Transfection with the Cu, Zn-Superoxide Dismutase Gene on Xanthine/Xanthine Oxidase-Induced Cytotoxicity in Fibroblasts from Rat Skin

Kohshi Nishiguchi,[1] Kazue Ishida,[2] Masanori Nakajima,[1] Tetsuo Maeda,[1] Fusao Komada,[1] Seigo Iwakawa,[2] Yusuke Tanigawara,[1] and Katsuhiko Okumura[1,3]

Received June 16, 1995; accepted January 2, 1996

Purpose. The effects of transfection with the human Cu, Zn-superoxide dismutase (hSOD)[4] gene on active oxygen-induced cytotoxicity in rat skin fibroblasts (FR) were studied for the purpose of developing the novel delivery system of hSOD using hSOD gene.

Methods. An expression plasmid for hSOD, pRc/RSV-SOD, was constructed and used to transfect FR cells. Xanthine (X)/xanthine oxidase (XO) system were used to generate active oxygen species. The effects of transfection with the hSOD gene on active oxygen-induced cytotoxicity were assessed by comparing the number of surviving cells and the level of lipid peroxidation in host and transformants after exposure to X/XO system.

Results. The cellular SOD activity in RSV-SOD cells transfected with pRc/RSV-SOD was significantly increased in comparison with host or RSV cells transfected with the pRc/RSV plasmid containing no hSOD gene as a control. Furthermore, Western blot analysis using an anti-hSOD antibody indicated the production of hSOD in RSV-SOD cells. On the other hand, although the numbers of surviving cells in both host and RSV-SOD cultures after exposure to X/XO system decreased in a time-dependent manner, the decrease in number of surviving RSV-SOD cells was less than that in host cells. In the presence of catalase, the decreases in number of surviving cells in both host and RSV-SOD cultures after exposure to the X/XO system were also less than those in the absence of catalase. However, the decreases in cell survival in RSV-SOD cultures were significantly less than those in host cells in the presence of catalase. Furthermore, the levels of lipid peroxidation in RSV-SOD cells exposed to the X/XO system in the presence or absence of catalase were lower than those in host cells. These results indicated that the increase in cellular SOD activity by transfection with the hSOD gene protects cells from oxidative stress.

Conclusions. Human SOD gene therapy may be useful for treatment of diseases in which oxidative tissue damage is produced.

KEY WORDS: human Cu, Zn-SOD; catalase; oxidative stress; gene therapy.

INTRODUCTION

Oxygen metabolites such as the superoxide anion (O_2^-) play an important role in many different pathophysiological processes including inflammation, rheumatoid arthritis, adult respiratory distress syndrome, aging, thermal injury, and carcinogenesis (1, 2). Cu, Zn-Superoxide dismutase (SOD), which catalyzes the dismutation of the toxic O_2^- into O_2 and H_2O_2, has been shown to play a pivotal role in protecting cells from free radical damage (3). The protective role of cellular SOD against oxidative stress has been demonstrated *in vitro* (4), and cultured cells with increased SOD activity were shown to be more resistant to oxidative stress (5). Recently, we observed the induction of endogenous cellular SOD during the accelerated wound healing at burn sites treated with epidermal growth factor (EGF) ointment in rats (6, 7). These findings suggest that therapeutic strategies to increase SOD activity may be useful for the treatment of diseases in which oxidative tissue damage is produced. However, although several attempts to extend the half-life of SOD in blood have been performed (8, 9), direct delivery of SOD into the intracellular space has not been reported. Such difficulty in delivery of macromolecules may also limit the clinical use of the other therapeutic proteins.

To solve such problems, we proposed the use of gene therapy as a new approach to deliver SOD into the intracellular space; i.e. if the cells themselves would be the site of synthesis of SOD by the direct transfer of the hSOD gene into the cells, high concentrations and long-lasting effects of SOD would be expected within the cells, and systemic side effects might be greatly reduced (10).

In the present study, to evaluate the possibilities of gene therapy with the hSOD gene for diseases related to oxidative stress, we investigated the effects of transfection with the hSOD gene on active oxygen-induced cytotoxicity generated enzymatically in rat skin fibroblasts (FR).

MATERIALS AND METHODS

Materials

Human Cu, Zn-SOD (hSOD) cDNA which was inserted into the *Bam*HI restriction site of the plasmid vector pUC119 and recombinant hSOD (3200 U/mg of protein) were kindly supplied by Asahi Chemical Ind. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), Opti-MEM and Lipofectin™, and xanthine oxidase were purchased from Flow Laboratories (Rockville, MD), Life Technologies (Grand Island, NY), and Boehringer Mannheim GmbH (Mannheim, Germany), respectively. *E. coli* HB101 competent cells, restriction endonucleases (*Hind* III and *Xba* I), and PCR primers were purchased from Takara Shuzo Co. (Kyoto, Japan). Trypsin solution, G418, trypan blue, catalase, xanthine and DAB were purchased from

[1] Department of Hospital Pharmacy, School of Medicine, Kobe University, 7-5-2, Kusunoki-cho, Chuo-ku, Kobe 650, Japan.

[2] Kobe Pharmaceutical University, 4-19-1, Motoyamakita-machi, Higashinada-ku, Kobe 658, Japan.

[3] To whom correspondence should be addressed.

[4] **ABBREVIATIONS** hSOD: Human Cu, Zn-superoxide dismutase; cDNA: Complementary DNA; FR cells: Rat skin fibroblast cell line; pRc/RSV: Eukaryotic expression plasmid vector utilizing the long terminal repeat from Rous sarcoma virus; pRc/RSV-SOD: pRc/RSV containing hSOD cDNA; RSV cells: FR cells transfected with pRc/RSV; RSV-SOD cells: FR cells transfected with pRc/RSV-SOD; FBS: Fetal bovin serum; G418: Geneticin disulfate; X: Xanthine; XO: Xanthine oxidase; O_2^-: superoxide anion; ·OH: Hydroxyl radical; DMEM: Dulbecco's modified Eagle's medium; PCR: Polymerase chain reaction; DAB: Diaminobenzidine; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis TBS: Tris-buffered saline TBA: Thiobarbituric acid; MDA: Malondialdehyde; EGF: Epidermal growth factor.

0724-8741/96/0400-0577$09.50/0

Wako Pure Chemicals (Osaka, Japan). All other chemicals were of reagent grade.

Cell Culture

The rat skin fibroblast cell line ATCC CRL 1213 (FR) was obtained from the American Type Culture Collection and was grown in DMEM containing 10% fetal bovine serum (FBS) and penicillin-streptomycin mixture (Bio Whittaker, Walkersville, MD) at 37°C in a humidified atmosphere of 5% CO_2.

Construction of Expression Plasmid Containing hSOD Gene

The expression plasmid for hSOD (pRc/RSV-SOD, Fig. 1) was constructed using a recombinant PCR technique; a Gene Amp PCR Reagent kit with Ampli Taq DNA polymerase (Perkin-Elmer Cetus Instruments, Norwalk, CT) was used for PCR. Briefly, recognition sites of the restriction endonucleases *Hind* III and *Xba* I were added to 5′ and 3′ ends of the hSOD cDNA (471 bp) by PCR using Primer I ($^{5'}$ AAAAGCTTATGGCGACGAAGGCCGTGTGC$^{3'}$) and Primer II ($^{5'}$AATCTAGAGAATGTTTATTGGGCGATCCC$^{3'}$), respectively. Both pRc/RSV (Invitrogen Co., San Diego, CA) and amplified DNA fragment (487 bp) containing hSOD cDNA were digested by *Hind* III and *Xba* I. Then, the fragments obtained were ligated using a DNA ligation kit (Takara Shuzo Co., Kyoto, Japan) and pRc/RSV-SOD (5593 bp) was constructed. We verified that the amplified DNA fragment inserted into pRc/RSV-SOD contained the hSOD cDNA by DNA sequencing (data not shown). The plasmid was prepared in large quantities in *E. coli* transformed by the alkaline lysis method. The purity of plasmid thus obtained was checked by electrophoresis on a 0.7% agarose gel.

Transfection

FR cells were transformed with pRc/RSV or pRc/RSV-SOD using the Lipofection method (11). Briefly, a mixture of Lipofectin™ (10 μg) and plasmid (5 μg) in Opti-MEM (final volume; 2 ml) was added directly to the surface of cells which were plated into culture dishes (60 mm in diameter, 1×10^5 cells) in DMEM supplemented with 10% FBS 24 h before transfection. After incubation for 5 h, DMEM with 10% FBS was added and the cells were cultured. G418 (final conc. 400 μg/ml) as the selection reagent for the transformants was added to the cells after 48 h. About two weeks later, the transformants were selected and used within 45 days after transfection for the experiments described below.

Determination of Cellular SOD Activity

Samples for determination of cellular SOD activity were prepared by a modification of the method described in our previous paper (7). Briefly, cells were separated and centrifuged, and the pellet was resuspended with distilled water (70 μl) and then left at −80°C for one hour. The frozen sample was allowed to stand at room temperature to disrupt the cells followed by centrifugation at 14,000 rpm for 10 min. Then SOD activity in the supernatant was measured by the nitrite method with hSOD protein as a standard.

Detection of hSOD Expression in Cells

Expression of hSOD in cells was detected by Western blot analysis using a modification of the method described in our previous paper (7). Briefly, the samples for determination of cellular SOD activity containing 50 μg of protein were subjected to 12.5 % SDS-PAGE. The proteins were then transferred onto polyvinylidene difluoride membranes (Millipore Co., Bedford, MA) at 120 mA for 5 h. The membranes were soaked in TBS containing 3% gelatin to block nonspecific binding. The signal of hSOD protein was visualized using the avidin-biotin complex method with anti-hSOD whole serum (The Bindingsite, Birmingham, UK), biotin-labeled purified rabbit anti-sheep IgG (EY Laboratories, San Mateo, CA), streptavidin-biotinylated horseradish peroxidase complex (Amersham International, plc, Bucks, UK), and DAB and hydrogen peroxide as the substrate. Recombinant hSOD protein was used as a standard.

Effects of Transfection with hSOD Gene on X/XO System

We used the X/XO system for production of active oxygen *in vitro* (12). The effects of transfection with hSOD gene on the X/XO system were evaluated as follows. Cells (2×10^5) were incubated in DMEM (2 ml) with 10% FBS in dishes (35-mm diameter) 24 h before exposure to the X/XO system. The medium was replaced with FBS-free medium (2 ml) with or without catalase (final conc. 3, 30 and 300 U/ml), and X (final conc. 0.25 and 0.5 mM) and XO (final conc. 2.5 and 5 mU/ml) were then added directly to each dish (host and RSV-SOD cells). After incubation at 37°C for various periods (3, 6 and 24 h), cell survival was assessed using the Trypan blue exclusion method. When host and RSV-SOD cells were treated with X or XO alone, the numbers of surviving cells of both types were not significantly different from control cells (data not shown).

Assay of Thiobarbituric Acid (TBA)-Reactive Substances in Cells

The cells were scraped with a rubber policeman after exposure to the X/XO system for 24 h, and centrifuged at 3,000 rpm for 5 min. The cell pellets were resuspended in 1.15% KCl (0.5 ml) and used for the assay of TBA-reactive substances in low actinic test tubes. Formation of TBA-reactive substances was measured with a lipid peroxide assay kit (Wako Pure Chemicals, Osaka, Japan), using malondialdehyde (MDA) according to Yagi *et al.* (13), and lipid peroxidation was expressed as MDA equivalents per cell.

Statistical Analysis

Statistical analysis of the data was performed with Student's *t*-test or the analysis of variance (ANOVA). Statistical significance was defined as a $p < 0.05$.

RESULTS

Firstly, the hSOD expression plasmid pRc/RSV-SOD, the structure of which is shown in Fig. 1, was constructed using recombinant PCR techniques.

RSV and RSV-SOD cells were obtained by transfection of FR cells with pRc/RSV and pRc/RSV-SOD, respectively.

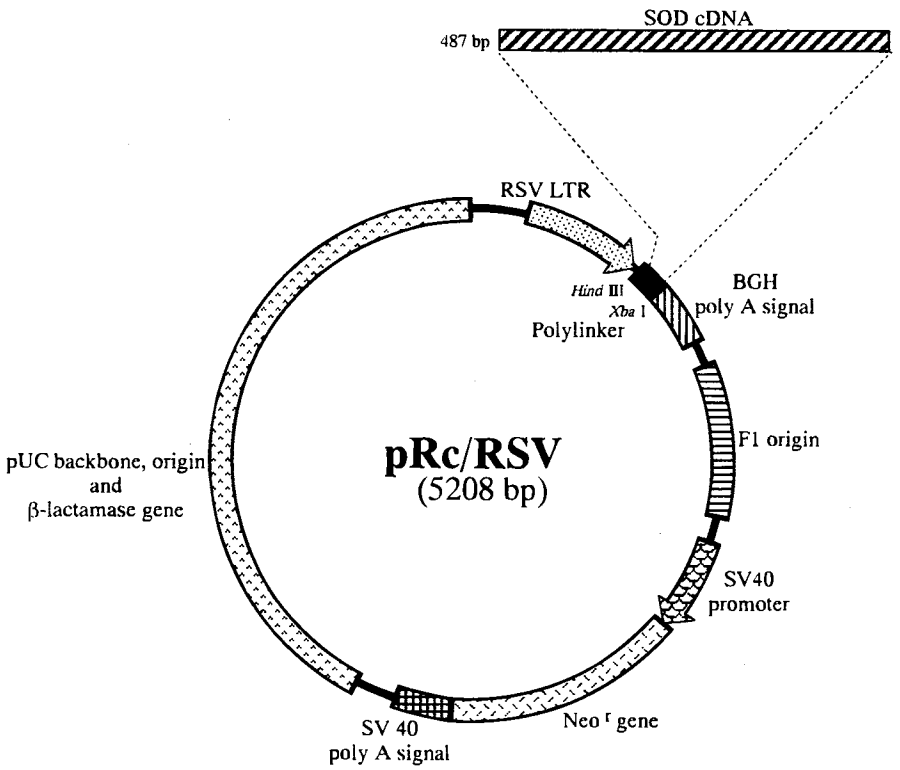

Fig. 1. Structure of pRc/RSV-SOD. ▨; SOD cDNA (487 bp), ▩; sequences from pUC119 including the β-lactamase gene and the Col E1 origin of DNA replication (2993–5208 bp), ▩; Rous sarcoma virus (RSV) promoter, ■; polylinker containing the recognition sites of *Hind* III and *Xba* I, ▥; BGH polyadenylation signal, ▤; F1 origin, ▨; SV 40 promoter, ▩; Neomycin resistance (Neo^I) gene, ▦; SV 40 polyadenylation signal.

The cellular SOD activities in host, RSV and RSV-SOD cells are shown in Fig. 2A. The cellular SOD activity in RSV-SOD cells was increased significantly (about 1.7-fold) relative to those in both host and RSV cells. However, no significant difference in the cellular SOD activity was observed between host and RSV cells. Furthermore, as shown in Fig. 2B, a band of the same size (approximately 16 kDa) as recombinant hSOD protein was observed only in RSV-SOD cells on Western blot analysis. As polyclonal rabbit antiserum for hSOD was used for these Western blots, other blots containing endogenous rat SOD were probably observed in host and RSV-SOD cells.

To assess the effects of transfection with the hSOD gene on oxidative stress, the numbers of surviving cells in host and RSV-SOD cultures after exposure to the X/XO system in the presence or absence of catalase were measured. The results are shown in Fig. 3. In the absence of catalase (Fig. 3A), cell survival was decreased not only in a time (3, 6, and 24 h)-dependent manner, but was also dependent on dose of X/XO (0.5 mM/5 mU/ml and 0.25 mM/2.5 mU/ml) in both cell types. Further, the decreases in number of surviving RSV-SOD cells at the indicated times were significantly less than those in host cells cultures. The decreases in cell survival in both cells types were also less in the presence than in the absence of catalase at 3, 30, and 300 U/ml. However, the decreases in number of surviving RSV-SOD cells at each dose of catalase were significantly less than those in host cells. These results indicate that RSV-SOD cells were more resistant to oxidative stress than host cells.

It has been reported that oxygen-derived free radicals attack cell membranes and lipid peroxidation is then initiated (14). Therefore, we assessed lipid peroxidation by determining the levels of TBA-reactive substances in cells. Figure 4 shows the levels of TBA-reactive substances in both host and RSV-SOD cells after various treatments. Data are expressed as MDA nmol per 10^5 cells. Although the MDA levels in host cells after exposure to the X/XO system in the absence and presence of catalase were elevated significantly in comparison with those in control cells (untreated host cells), no elevation in level of MDA in RSV-SOD cells was observed. Further, the MDA levels in RSV-SOD cells after exposure to the X/XO system in the absence of catalase were significantly lower than those in host cells. These results indicate that lipid peroxidation in RSV-SOD cells occurred to a lesser extent that in host cells.

DISCUSSION

We reported previously that marked improvement and accelerated synthesis of Cu, Zn-SOD were found in burned tissue following treatment with EGF ointment in rats (6, 15, 16), and that accelerated transcription of the endogenous Cu, Zn-SOD gene by EGF was observed *in vitro* using rat skin fibroblasts exposed to enzymically-generated active oxygen species (7). The protective role of endogenous cellular SOD against oxidative stress, and the inhibition of oxygen-induced cytotoxicity by the supplementation of SOD protein into the intracellular space have also been reported in cultured cells (4,

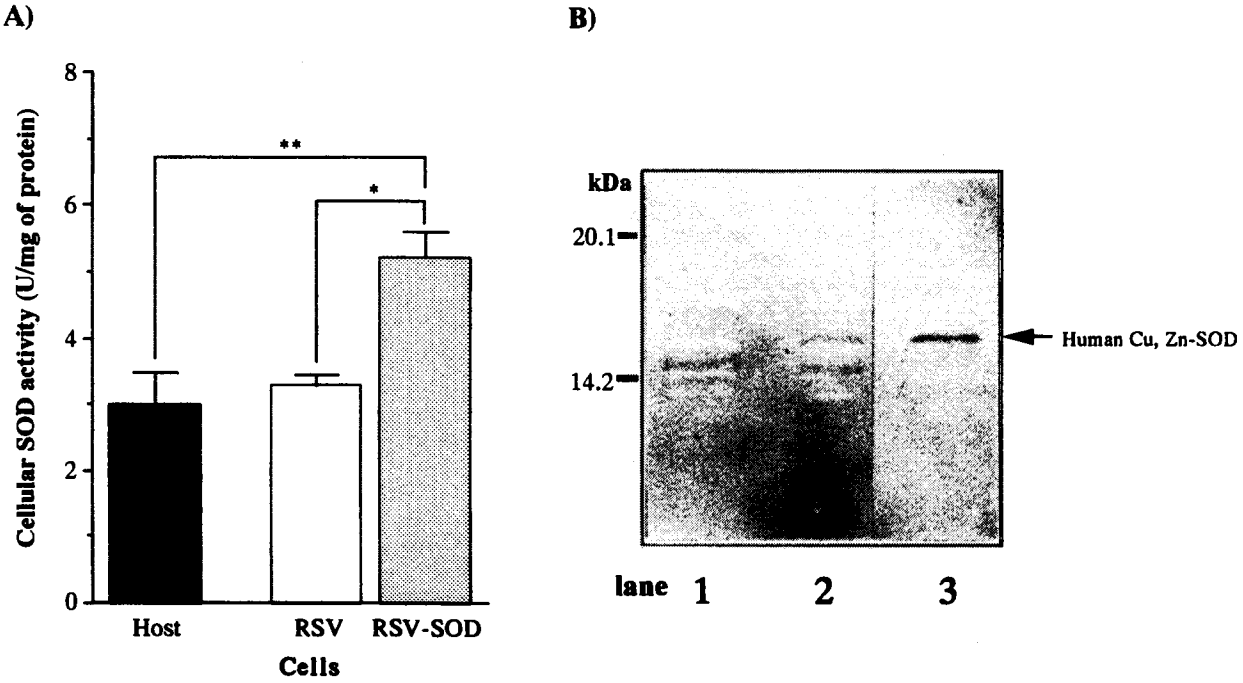

Fig. 2. Determination of cellular SOD activity (A) and detection of the expression of human Cu, Zn-SOD protein (B) in FR cells. A) Determination of cellular SOD activity: ■; host cells (untransfected FR cells), □; RSV cells (FR cells transfected with pRc/RSV), ▨; RSV-SOD cells (FR cells transfected with pRc/RSV-SOD). Bars represent means ± SE (n = 3). Statistical analysis of the data was performed with ANOVA (*$p < 0.05$ and **$p < 0.01$). B) Western blot analysis for hSOD protein: Each sample (50 μg of protein) was applied to SDS-PAGE for immunoblot analysis: lane 1; host cells, lane 2; RSV-SOD cells, and lane 3; rhSOD (standard).

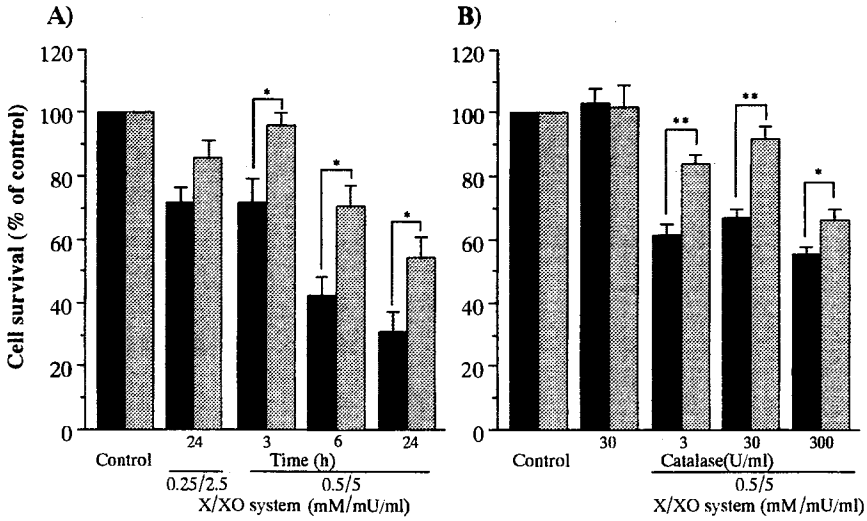

Fig. 3. Effects of transfection with hSOD gene against X/XO-induced cytotoxicity in the absence (A) or presence (B) of catalase. ■; host cells, ▨; RSV-SOD cells. Bars represent means ± SE (n = 3–6). Statistical analysis of the data was performed with Student's *t*-test (*$p < 0.05$ and **$p < 0.01$). Control; untreated cells. A) Numbers of surviving cells were counted at the indicated times after exposure to the X/XO (0.5 mM/5 mU/ml or 0.25 mM/2.5 mU/ml) system. B) The numbers of surviving cells were counted 24 h after exposure to the X/XO (0.5 mM/5 mU/ml) system in the presence of the indicated doses of catalase (3, 30, and 300 U/ml).

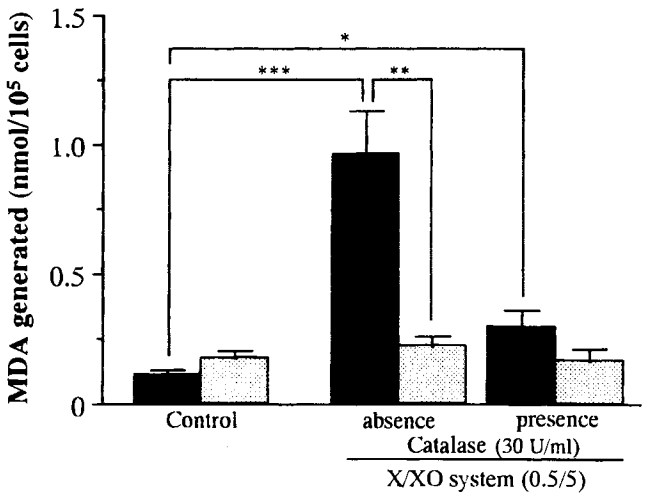

Fig. 4. Comparison of TBA-reactive substances between host and RSV-SOD cells. TBA-reactive substances were assayed in host and RSV-SOD cells 24 h after exposure to the X/XO (0.5 mM/5 mU/ml) system in the absence or presence of catalase (30 U/ml). ■; host cells and ▨; RSV-SOD cells. Control; untreated cells. Bars represent means ± SE (n = 3–9). Statistical analysis of the data was performed with Student's *t*-test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$).

5). These findings suggest that therapeutic regimens which increase cellular SOD activity may be useful in the treatment of diseases in which oxidative tissue damage is produced. However, although several attempts to extend the half-life of SOD in blood have been made (8, 9), studies of systems to deliver SOD protein into the intracellular space have been limited. We supposed that such a delivery problem might also limit the clinical use of the other therapeutic proteins. So, we now propose the use of therapeutic gene delivery as a new approach to deliver SOD protein into the intracellular space. Evans *et al.* (10) suggested that as the cell itself would be the site of synthesis of therapeutic protein by the direct transfer of a therapeutic gene into the cells, the highest concentration of therapeutic protein and long-lasting effects would be within the cells, and the side effects should be greatly reduced. In the present study, therefore, to evaluate the possibilities of gene therapy with the hSOD gene for diseases related to oxidative stress, we investigated the effects of cellular SOD activity following transfection with the hSOD gene on enzymically generated active oxygen-induced cytotoxicity in rat skin fibroblasts.

Initially, we constructed the plasmid pRc/RSV-SOD as a vector to deliver the hSOD gene into the cells (Fig. 1). The structure of pRc/RSV-SOD was confirmed by digestion with restriction endonucleases (*Hind* III and *Xba* I) and Southern blot analysis using hSOD cDNA as a probe (data not shown). As shown in Fig. 2A, cellular SOD activity in RSV-SOD cells was increased relative to that in host cells. A band of the same size (approximately 16 kDa) as recombinant hSOD protein observed in RSV-SOD cells also indicated the expression of hSOD following transfection of FR cells with pRc/RSV-SOD (Fig. 2B). These findings suggest that the increase in cellular SOD activity in RSV-SOD cells was due to the expression of hSOD protein as a result of transfection with pRc/RSV-SOD and that this plasmid may be a useful vector for delivery of the hSOD gene into cells. We also used the X/XO system, which produces superoxide anion and hydrogen peroxide (12), as a system for the enzymatic generation of extracellular oxidative stress. This form of oxidative stress mimics the action of phagocytic leukocytes in inflammation which produce large amounts of active oxygen close to the surfaces of target cells (17). As there were no significant differences in cell survival between host and RSV cells 24 h after exposure to the X/XO system (data not shown), transfection itself did not appear to influence the antioxidant activity of FR cells. The significant increase in cell survival in RSV-SOD cells in comparison with host cells in the absence of catalase (Fig. 3A) indicates that the increase in cellular SOD activity achieved by transfection with the hSOD gene had a protective effect against oxidative stress. Further, the increase of cellular SOD activity in RSV-SOD cells was increased by about 1.7-fold relative to host cells consistent with previous results (7), so these results also indicated that the increase of cellular SOD activity was involved in the biochemical mechanism of the EGF-induced healing process following injury. However, Amstad *et al.* reported that the balance of antioxidant defense functions was more important than simple adjustments of individual parts of the system in a study using cells transfected with the SOD gene (18). Further, it has been reported that under physiological conditions enzymes such as catalase which dismute H_2O_2 are present at high levels in the body (19). In the presence of catalase, a significant increase in survival of RSV-SOD cells in comparison with host cells was observed (Fig. 3B), indicating that the increase in cellular SOD activity achieved by transfection with the hSOD gene protected cells from oxidative stress in vivo. However, a decrease in protection against oxidative stress was observed in the presence of excess catalase (300 U/ml). Although it was speculated that these effects might be ascribed to the cytotoxicity by the excess of catalase as described by Speraza *et al.* (20), the detailed mechanisms underlying our results remain unclear. Thus, further studies are necessary.

Oxygen-derived free radicals, mainly superoxide anion and hydroxyl radicals, attack cell membranes, and then lipid peroxidation is initiated (14). In the X/XO system used in this study, large amounts of O_2^- and H_2O_2 are generated close to the cell surface. O_2^- also reduces Fe^{3+} to Fe^{2+}, and Fe^{2+} can react with excess H_2O_2 to form the more toxic species ·OH (21). On the other hand, extracellular H_2O_2 can penetrate cell membranes because it is electroneutral. H_2O_2 within cells is detoxified principally by the glutathione redox cycle and endogenous catalase. However, intracellular excess of H_2O_2 causes O_2^- generation (possibly through reaction with Fe^{3+}), and the generated O_2^- reduces Fe^{3+} to Fe^{2+} (4). The Fe^{2+} thus generated could then react with excess H_2O_2 to form ·OH. Thus, to assess the effects of transfection with the hSOD gene on lipid peroxidation in cells after exposure to active oxygen species, we determined the levels of TBA-reactive substances in host and RSV-SOD cells after exposure to the X/XO system in the absence and presence of catalase (Fig. 4). The elevated level of TBA-reactive substances in host cells after exposure to the X/XO system in both the absence and presence of catalase, but not in RSV-SOD cells, indicates that the cytoprotective effect of transfection with the hSOD gene against extracellular oxidative stress may be based, at least in part, on a reduction in lipid peroxidation within cells.

In conclusion, the present results showed that the increase in cellular SOD activity achieved by transfection with the hSOD

gene protects cells from the cytotoxic effects of extracellular oxidative stress. Our findings further suggest that hSOD gene therapy may be useful in treatment of pathophysiological conditions such as inflammation, adult respiratory distress syndrome and rheumatoid arthritis, which are related to superoxide release, and for hereditary diseases such as familial amyotrophic lateral sclerosis, caused by mutations in the Cu, Zn-SOD gene (22). However, to use gene therapy clinically, bio-pharmaceutical studies to achieve appropriate delivery of the therapeutic gene into targeted tissues are necessary.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research (B) (No. 04454543) from the Ministry of Education, Science and Culture, Japan. We are grateful to Asahi Chemical Industries for providing recombinant human Cu, Zn-SOD and human Cu, Zn-SOD cDNA.

REFERENCES

1. C. E. Cross, B. Halliwell, E. T. Borish, W. A. Pryor, B. N. Ames, R. L. Saul, J. M. McCord, and D. Massaro. Oxygen radicals and human disease. *Ann. Intern. Med.* **107**:526–545 (1987).
2. C. G. Cochrane, R. Spragg, and S. D. Revak. Pathogenesis of the adult respiratory distress syndrome. Evidence of oxidant activity in bronchoalveolar lavage fluid. *J. Clin. Invest.* **71**:754–761 (1983).
3. I. Fridovich. Superoxide dismutase. *J. Biol. Chem.* **264**:7761–7764 (1989).
4. H. Hiraishi, A. Terano, T. Sugimoto, T. Harada, M. Razandi, and K. J. Ivey. Protective role of intracellular superoxide dismutase against extracellular oxidants in cultured rat gastric cells. *J. Clin. Invest.* **93**:331–338 (1994).
5. B. A. Markey, S. H. Phan, J. Varani, U. S. Ryan, and P. A. Ward. Inhibition of cytotoxicity by intracellular superoxide dismutase supplementation. *Free Radicals Biol. Med.* **9**:307–314 (1990).
6. Y. Kiyohara, K. Nishiguchi, F. Komada, S. Iwakawa, M. Hirai, and K. Okumura. Cytoprotective effects of epidermal growth factor (EGF) ointment containing protease inhibitor, nafamostat, on tissue damage at burn sites in rats. *Biol. Pharm. Bull.* **16**:1146–1149 (1993).
7. K. Nishiguchi, Y. Kiyohara, F. Komada, S. Iwakawa, and K. Okumura. Effect of epidermal growth factor on Cu, Zn-superoxide dismutase expression in cultured fibroblasts from rat skin. *Pharm. Res.* **11**:1244–1249 (1994).
8. T. Ogino, M. Inoue, Y. Ando, A. Awai, H. Maeda, and Y. Morino. Chemical modification of superoxide dismutase: Extension of plasma half life of the enzyme through its reversible binding to the circulating albumin. *Int. J. Pept. Protein Res.* **32**:153–159 (1988).
9. P. S. Pyatak, A. Abuchowski, and F. F. Davis. Preparation of a polyethylene glycol: superoxide dismutase adduct, and an examination of its blood circulation life and anti-inflammatory activity. *Res. Commun. Chem. Pathol. Pharmacol.* **29**:113–127 (1980).
10. C. H. Evans, and P. D. Robbins. Gene therapy for Arthritis. In J. A. Wolff (eds.), *Gene Therapeutics: Methods and Applications of Direct Gene Transfer,* Birkhäuser, Boston, 1994, pp. 320–343.
11. P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**:7413–7417 (1987).
12. I. Fridovich. Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. *J. Biol. Chem.* **245**:4053–4057 (1970).
13. K. Yagi. A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med.* **15**:212–216 (1976).
14. I. S. Ayene, C. Dodia, and A. B. Fisher. Role of oxygen in oxidation of lipid and protein during ischemia/reperfusion in isolated perfused rat lung. *Arch. Biochem. Biophys.* **296**:183–189 (1992).
15. K. Okumura, Y. Kiyohara, F. Komada, S. Iwakawa, M. Hirai, and T. Fuwa. Improvement in wound healing by epidermal growth factor (EGF) ointment I. Effect of nafamostat, gabexate, or gelatin on stabilization and efficacy of EGF. *Pharm. Res.* **7**:1289–1293 (1990).
16. Y. Kiyohara, F. Komada, S. Iwakawa, M. Hirai, T. Fuwa, and K. Okumura. Improvement in wound healing by epidermal growth factor (EGF) ointment II: Effect of protease inhibitor, nafamostat, on stabilization and efficacy of EGF in burn. *J. Pharmacobio-Dyn.* **14**:47–52 (1991).
17. R. H. Simon, C. H. Scoggin, and D. Patterson. Hydrogen peroxide causes the fatal injury to human fibroblasts exposed to oxygen radicals. *J. Biol. Chem.* **256**:7181–7186 (1981).
18. P. Amstad, A. Peskin, G. Shah, M. E. Mirault, R. Moret, I. Zbinden, and P. Cerutti. The balance between Cu, Zn-superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to oxidative stress. *Biochemistry* **30**:9305–9313 (1991).
19. M. J. Goebel, N. Rudolph, and S. L. Wong. Developmental patterns of antioxidant defense mechanisms in human erythrocytes. *Pediatr. Res.* **26**:366–369 (1989).
20. M. J. Speranza, A. C. Bagley, and R. E. Lynch. Cells enriched for catalase are sensitized to the toxicities of bleomycin, adriamycin, and paraquat. *J. Biol. Chem.* **268**:19039–19043 (1993).
21. B. Halliwell, and J. M. C. Gutteridge. The importance of free radicals and catalytic metal ions in human diseases. *Mol. Aspects Med.* **8**:89–193 (1985).
22. D. R. Rosen, T. Siddique, D. Patterson, D. A. Figlewicz, P. Sapp, A. Hentati, D. Donaldson, J. Goto, J. P. O'Regan, H.-X. Deng, Z. Rahmani, A. Krizus, D. Mck.- Yasek, A. Cayabyab, S. M. Gaston, R. Berger, R. E. Tanzi, J. J. Halperin, B. Herzfeldt, R. V. B. Bergh, W.-Y. Hung, T. Bird, G. Deng, D. W. Mulder, C. Smyth, N. G. Laing, E. Soriano, M. A. Pericak-Vance, J. Haines, G. A. Rouleau, J. S. Gusella, H. R. Horvitz and R. H. Brown, Jr. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **362**:59–62 (1993).