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Research Paper

Regulation of Exogenous Gene Expression by Superoxide

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Purpose. Regulation of gene expression after gene introduction is a problematic aspect of gene therapy. Transcription regulates gene-specific transcriptional factors, which bind to regulatory regions in the promoter. The cytomegalovirus long terminal repeat (CMV-LTR) has a TPA response element (TRE) as a binding site for activator protein 1 (AP-1), which is induced by oxidative stress. The purpose of this study was to regulate exogenous gene expression in a vector with CMV-LTR using oxygen radicals.
Methods. We used two plasmids (1) pQBI25 encoding CMV-LTR and red-shift green fluorescent protein (rsGFP) cDNA and (2) pRc/CMV-SOD encoding CMV-LTR and human Cu, Zn-superoxide dismutase (SOD) cDNA. FR cells were transfected with pQBI25 (FR-pQBI25 cells), and L2 cells were transfected with pRc/CMV-SOD (L2-pRc/CMV-SOD cells). Each type of cell was exposed to oxygen radicals using paraquat for 24 h. Levels of *c-fos*, *c-jun* and *rsGFP* mRNAs were determined using reverse transcription polymerase chain reaction (RT-PCR). Levels of rsGFP protein were measured by fluorometry. Total SOD activity was measured using the nitrite method.
Results. Levels of *c-fos*, *c-jun* (AP-1 composition protein) and *rsGFP* mRNA were induced significantly by oxygen radical exposure in FR-pQBI25 cells. A positive correlation was observed between levels of *c-fos* mRNA and *rsGFP* mRNA and also between levels of *c-jun* mRNA and *rsGFP* mRNA. Levels of rsGFP protein were also induced significantly. Total SOD activity was induced significantly by oxygen radical exposure in L2-pRc/CMV-SOD cells.
Conclusions. This study suggests that gene expression driven by the CMV- LTR promoter may be regulated by oxygen radicals.

KEY WORDS: AP-1; cytomegalovirus; gene regulation; long terminal repeat; superoxide.

INTRODUCTION

Oxidative stress (e.g., superoxide) is associated with lipid peroxidation, several cancers, DNA mutation and ischemic-reperfusion disorder (1–3). Cu, Zn-superoxide dismutase (SOD), which catalyzes and eliminates superoxide, is approved in France and is now being investigated as a polyethylene glycol–SOD conjugate for clinical use (4,5). Generally, peptide and protein drugs, including SOD, have problems in drug delivery (low absorption, difficult targeting, fast elimination, etc.). Therefore, gene therapy is a novel drug delivery system for peptide or protein drugs. Previously, we reported *ex vivo* SOD gene delivery using a eukaryotic plasmid vector to skin fibroblast and lung epithelial-like cells and efficacy in *in vitro* experimental models (6,7). And also we reported anti-inflammatory effects of Cu, Zn-superoxide dismutase delivered by genetically modified cells on carrageenin-induced paw edema and cold-induced skin edema in rats *in vivo* (7,8).

Gene therapy has been used in several birth defects, such as adenosine deaminase deficiency or cancer; however, at present gene therapy is problematic, such as in regulation of gene expression after gene introduction (9,10). Inazawa et. al. reported on the expression of a eukaryotic plasmid vector with a MMTV promoter and 20K-growth hormone-regulated adrenocorticosteroids (11).

Regulation of gene expression in eukaryotic cells is crucial for maintenance of cellular homeostasis. The regulation of gene expression can occur in multiple steps. The overwhelming majority of regulatory events occur at the level of transcription. These transcriptional processes regulate *cis*- and *trans*-acting factors. Transcription factors that act as *trans*-acting factors are basal transcription factors, which are ubiquitous and recruit the RNA polymerase II multi-protein complex to the minimal promoter. Gene-specific transcription factors activate or repress basal transcription.

A eukaryotic expression plasmid vector must have a promoter region and these promoter are usually sequences of

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viral long terminal repeats, i.e., cytomegalovirus (CMV), rous sarcoma virus, or mouse mammary tumor virus. These viral promoters are affected by several transcription factors, and the level of gene expression is regulated in genetically modified cells. The CMV promoter is controlled by activator protein 1 (AP-1) because this promoter has a 12-*O*-tetradecanoyl phorbor-13-acetate (TPA) response element (TRE) (12).

Paraquat, which is used as an organic phosphorus insecticide, generates oxygen radicals. Thus, paraquat has been used as a model compound to induce oxidative stress in experimental system *in vitro* and *in vivo*. Li and Sun reported that AP-1 as a transcription factor was induced by paraquat (13). Many transcription factors are heterodimers. For example, AP-1 is composed of two transcription factors, c-Fos and c-Jun (14,15). The pQBI25 vector has a CMV promoter and a red-shift green fluorescent protein (rsGFP) cDNA. The pRc/CMV-SOD vector also has a CMV promoter and human SOD cDNA. In transformants with the pQBI25 and pRc/CMV-SOD vector, the level of rsGFP and SOD expression increase with the induction of AP-1 by oxidative stress, respectively. Therefore, in this study, we examined whether levels of *c-fos*, *c-jun*, *rsGFP* mRNAs, rsGFP protein and total SOD activity are induced by paraquat exposure *in vitro*. We also examined the association between levels of *c-fos* and *c-jun* mRNAs as AP-1 constituent expression, and the level of *rsGFP* mRNA. Our results indicated that reactive oxygen species mediated activation of AP-1 regulated exogenous gene expression, and thus the optimization of gene therapy may provide.

MATERIALS AND METHODS

Materials

Recombinant human SOD (3,200 U/mg of protein) was kindly supplied by Asahi Chemical (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin–streptomycin mixture were purchased from Wako Pure Chemical Industries (Osaka, Japan), Flow Laboratories (MD, USA), ICN Biomedicals (OH, USA), Bio Whittaker (ND, USA), respectively. RT-PCR kit (Ready To Go RT-PCR beads) and first strand cDNA synthesis kit (Ready To Go You Prime First Strand Beads) were purchased from Amersham Pharmacia Biotech (NJ, USA). *Taq* polymerase, rsGFP containing eukaryotic expression vector (pQBI25) and recombinant GFP were purchased from Takara Co. (Tokyo, Japan). Ham's F12 nutrient medium, Opti-MEM, paraquat and Geneticin sulfate (G418) were purchased from Sigma Chemical (NJ, USA), Gibco (NY, USA), respectively. PCR primers for *c-fos*, *c-jun*, *rsGFP* and *β-actin* mRNAs were purchased from Funakoshi (Tokyo, Japan), Sawady Technology (Tokyo, Japan), respectively. All other chemicals were of reagent grade.

Cell Cultures

Rat skin fibroblast cell lines (FR cells, ATCC CCL 1213) and rat lung epithelial-like cell lines (L2 cells, ATCC CCL 149) were obtained from American Type Culture Collection. FR and L2 cells were grown in DMEM containing 10% FBS, 100 units/mL penicillin and 100 μg/ml streptomycin (penicillin–streptomycin mixture) and Ham's F12 nutrient medium containing 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (penicillin–streptomycin mixture), respectively, at 37°C in a humidified atmosphere of 5% CO_2.

Preparations of Stable Transformants

FR cells were transfected with pQBI25 by lipofection (TransFast™ transfection reagent, Promega, WI, USA). L2 cells were transfected with pRc/CMV-SOD by lipofection (Lipofectin™ reagent, Life Technologies, MD, USA). After 48 h, resistant clones were selected in medium containing G418 (final concentration 400 μg/ml) for 2 weeks. Single clones of each transfected cell were used in this study. FR-pQBI25 cells (FR cells transfected with pQBI25) were grown in same condition of FR cells. L2-pRc/CMV-SOD cells (L2 cells transfected with pRc/CMV-SOD) were grown in same condition of L2 cells.

Quantification of the Levels of *rsGFP*, *c-fos* and *c-jun* mRNA

After 24 h of paraquat treatment, total RNA were extracted from each culture using a total RNA extraction kit (RNeasy Mini Kit, Qiagen, Hilden, Germany). RT-PCR for *β-actin* and *rsGFP* mRNAs were performed using an RT-PCR kit (Ready To Go RT-PCR beads), and those for *c-fos* and *c-jun* mRNAs were performed using a first strand cDNA synthesis kit (Ready To Go You Prime First Strand Beads) and *Taq* polymerase. The levels of these mRNAs were quantified by optical density of their bands on agarose gels using NIH Image (National Institutes of Health, NJ, USA), and the ratio of the expression levels of *rsGFP*, *c-fos* and *c-jun* mRNAs was normalized relative to that of *β-actin* mRNA. We previously confirmed that RT-PCR kinetic curves of these ratios of mRNAs did not reach a plateau (16). The RT-PCR kinetic curves of the ratios of the levels of *c-fos*, *c-jun* and *rsGFP* mRNAs/*β-actin* mRNA in this study could be determined quantitatively at least up to a ratio of approximately 3. Therefore, we could estimate the levels of *rsGFP*, *c-fos* and *c-jun* mRNA expression quantitatively.

Determination of the Levels of SOD Activity

Cells were centrifuged and separated, and then the pellet was resuspended with distilled water (70 μl) and left at −80°C for 1 h. The frozen sample was allowed to stand at room temperature to disrupt the cells, followed by centrifugation at 14,000 rpm for 10 min. Total SOD activity in the supernatant was measured using the nitrite method (17). Cellular protein levels were determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, CA, USA).

Determination of the Levels of rsGFP Protein

Cells were centrifuged and separated, and the pellet was resuspended with 0.1% Tween solution and then left at −80°C for 1 h. The frozen sample was allowed to stand at room temperature to disrupt the cells followed by centrifugation at 14,000 rpm for 10 min. The protein level of rsGFP in supernatant was measured by the fluorometer (EX 510 nm and EM 490 nm) with GFP protein as a standard. Cellular protein levels were also determined using a Bio-Rad Protein Assay Kit.

In vitro Experiment System of Paraquat Exposure in FR-pQBI25 Cells

Aliquots of 1.3×10^6 FR-pQBI25 cells were seeded in 60-mm tissue culture dishes containing growth medium. After 24 h, the incubation medium was replaced with each test medium containing the final concentrations of 5, 20 and 50 μM of paraquat. After 24 h of paraquat exposure, total RNA were extracted from each culture and the levels of *rsGFP*, *c-fos* and *c-jun* mRNA were quantified. In parallel, cytosolic proteins were extracted from each culture and the levels of rsGFP protein were determined.

In vitro Experiment System of Paraquat Exposure in L2-pRc/CMV-SOD Cells

Aliquots of 2.0×10^5 L2-pRc/CMV-SOD cells were seeded in 35-mm tissue culture dishes containing growth medium. After 24 h, the incubation medium was replaced with each test medium containing the final concentration of 100 μM paraquat (17). The levels of SOD activity were determined in the cells.

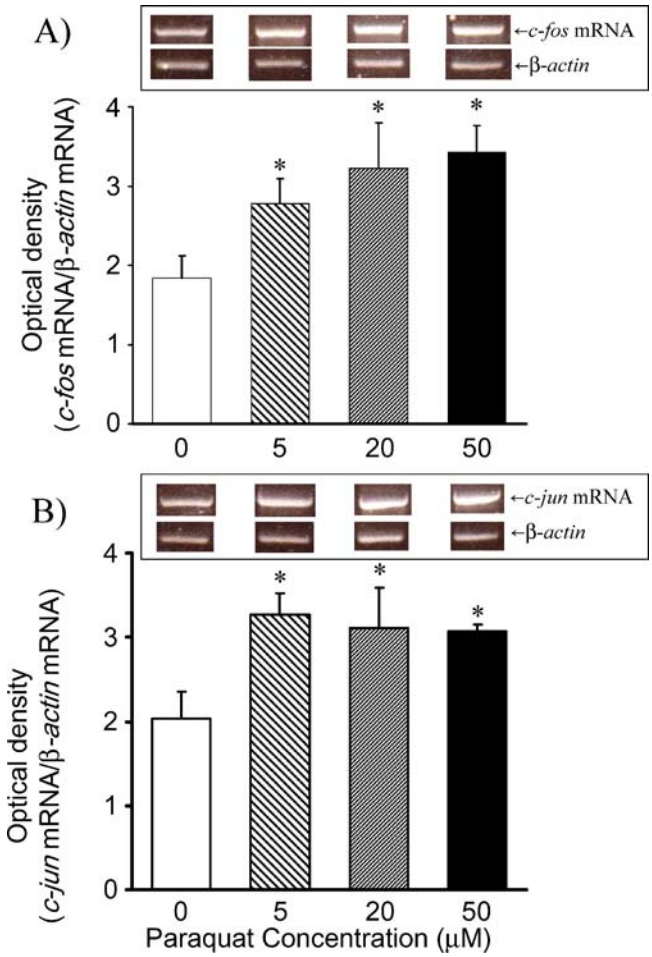

Fig. 1. Effects of paraquat treatment on *c-fos* and *c-jun* mRNA expression in FR-pQBI25 cells. (A) Levels of *c-fos* mRNA expression. *Upper panel* shows representative blots of *c-fos* mRNA and *β-actin* mRNA. (B) Levels of *c-jun* mRNA expression. *Upper panel* shows representative blots of *c-jun* mRNA and *β-actin* mRNA. Each *bar* represents mean±SEM (n = 5–9). *$p < 0.05$ compared with control condition.

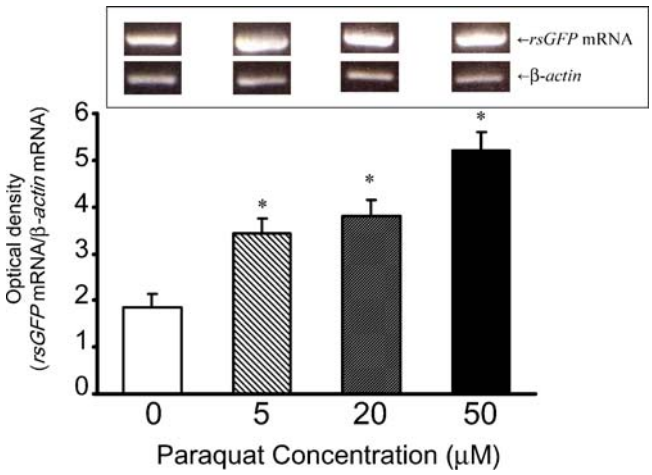

Fig. 2. Effects of paraquat treatment on *rsGFP* mRNA expression in FR-pQBI25 cells. *Upper panel* shows representative blots of *rsGFP* mRNA and *β-actin* mRNA. Each *bar* represents mean±SEM (n = 5–9). *$p < 0.05$ compared with control condition.

Statistical Analysis

Statistical analysis was performed by analysis of variance (ANOVA). A level of $p<0.05$ was considered statistically significant.

RESULTS

Inductions of *c-fos* and *c-jun* mRNAs

We examined the effect of paraquat on levels of *c-fos* and *c-jun* mRNA expression in FR-pQBI25 cells. Both mRNA levels were increased significantly at each concentration of paraquat treatment. The levels of *c-fos* mRNA were increased 1.5- to 1.9-fold in comparison with that in control conditions (no paraquat treatment) and also the induction of *c-fos* mRNA in a paraquat-concentration-dependent manner was observed (Fig. 1). The *c-jun* mRNA level was increased 1.5- to 1.6-fold in comparison with that in control conditions, although the induction of *c-jun* mRNA levels did not occur in a paraquat-concentration-dependent manner.

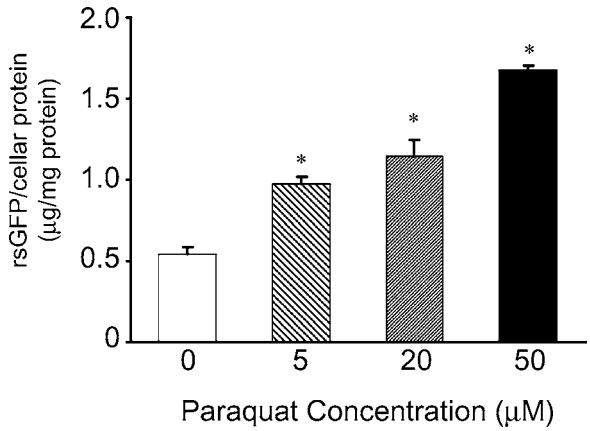

Fig. 3. Effects of paraquat treatment on rsGFP protein expression in FR-pQBI25 cells. Each *bar* represents mean±SEM (n = 8–9). *$p <$ 0.05 compared with control condition.

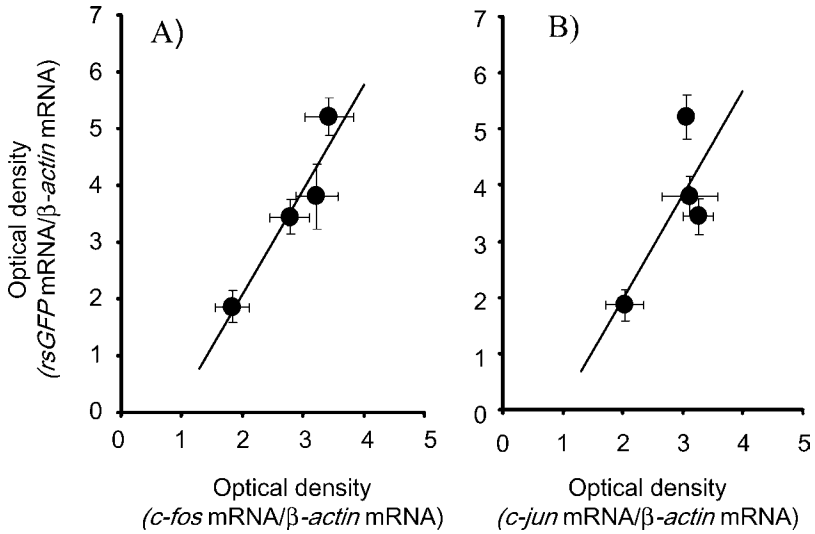

Fig. 4. Correlation (a) between *rsGFP* mRNA and *c-fos* mRNA, and the correlation (b) between *rsGFP* mRNA and *c-jun* mRNA expression in FR-pQBI25 cells. Correlation coefficients for (a) and (b) by linear approximation least-squares method were 0.9508 and 0.7552, respectively. Each *point* represents mean±SEM (n = 5–9).

Induction of *rsGFP* mRNA and Protein

Figures 2 and 3 show levels of *rsGFP* mRNA and rsGFP protein expression after paraquat treatment, respectively. The level of *rsGFP* mRNA expression was increased 1.9- to 2.9-fold in comparison with that in control conditions and the expression of *rsGFP* mRNA was paraquat-concentration dependent. The levels of rsGFP protein expression increased by 1.8- to 3.1-fold in comparison with that in control conditions; this increase was paraquat-concentration dependent.

Correlation between *c-fos* mRNA, *c-jun* mRNA and *rsGFP* mRNA Expression

We examined whether the paraquat-induced *c-fos* and *c-jun* mRNA expression affected induction of *rsGFP* mRNA expression. Figure 4a, b show the relationship between levels of *c-fos* mRNA and *rsGFP* mRNA expression, and the relationship between levels of *c-jun* mRNA and *rsGFP* mRNA, respectively. A positive correlation was observed between levels of *c-fos* mRNA and *rsGFP* mRNA, and was observed between levels of *c-jun* mRNA and *rsGFP* mRNA on the each concentration of paraquat treatments.

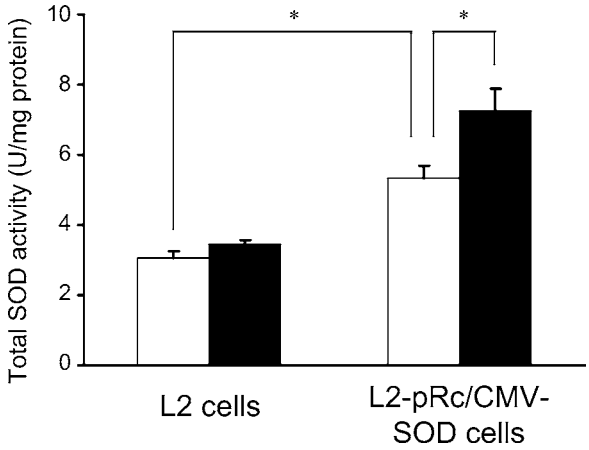

Fig. 5. Effects of paraquat treatment on SOD activity in L2-pRc/CMV-SOD cells. □, no treatment; ■, 100 μM paraquat. Each *bar* represents mean±SEM (n = 3–12). *$p < 0.05$.

Induction of Total SOD Activity

Figure 5 shows the levels of total SOD activity in L2 and L2-pRc/CMV-SOD cells with or without paraquat treatment. The level of total SOD activity in L2-pRc/CMV-SOD cells increased 1.8-fold in comparison with normal L2 cells in the control condition (no paraquat treatment). Total SOD activity was not increased by treatment with paraquat in normal L2 cells; however, significant induction of total SOD activity was observed in L2-pRc/CMV-SOD cells by paraquat treatment. The level of total SOD activity in L2-pRc/CMV-SOD cells was increased by 1.4-fold in comparison with that in the control condition.

DISCUSSION

In this study, we examined the effect of paraquat on the expression of rsGFP cDNA transfected rat skin fibroblasts and human SOD cDNA transfected rat lung epithelial-like cells *in vitro*.

We confirmed that, after 6 months, FR-pQBI25 cells expressed rsGFP using fluorescent microscopy and we detected a rsGFP mRNA-derived single band using ethidium bromide stained agarose gel electrophoresis after RT-PCR. L2-pRc/CMV-SOD cells also showed long-term (over 6 months) expression after transfection using the lipofection method (18).

AP-1 is comprised of members of the FOS and JUN family and is in the family of basic domain/leucine zipper transcriptional factors that have been characterized for their specific binding to TRE (19,20). The amount of AP-1 is regulated by the level of *fos* and *jun* mRNA species. AP-1 is activated by mitogen-activated protein kinase families (21). AP-1 regulates the expression of many genes, including those

encoding collagenase, stromelysin, cyclin D, TGF-1 beta and many cytokines, by binding to TREs in the promoters of these genes (22). Expression of c-JUN and c-FOS is induced by phorbol esters such as TPA (23,24). Furthermore, AP-1 has been shown to be induced by paraquat, H_2O_2, UV-A, UV-C, dioxin, hyperglycaemia, substance P and others (25–32). The induction of *c-fos* and *c-jun* mRNA in FR cells is suggested to be caused by reactive oxygen from paraquat, and these mRNAs were induced AP-1 activity.

Paraquat and H_2O_2 induced not only AP-1 but also nuclear factor kappaB (NF-κB) (33,34). Sequence analysis of the mouse GPx and CAT genes revealed putative binding motifs for NF-κB and AP-1 which were actively involved in the upregulation of the GPx and CAT in response to oxidative stress. NF-κB and AP-1 are important mediators of redox-responsive gene expression. The relative involvement for inductions and effects of other transcriptional factors or AP-1 family members remains to be established.

In this study, we looked at paraquat induction of AP-1 family members, *c-jun* and *c-fos* mRNA 24 h after treatment and reported a 1.5- to 1.9-fold increase. Both *c-jun* and *c-fos* mRNA are early response genes and are most often induced within 15 to 30 min after exposure to stimuli (13). Gene expression is usually return to uninduced levels within the first few hours. On the other hand, our data showed that gene expression of AP-1 family members can occur later and is sustained for a long-term period. We investigated inductions of *c-jun* and *c-fos* expression 4 h after paraquat treatment in secretable SOD (SOD conjugated with interleukin-2 signal peptide) overexpressed FR cells. But no inductions of *c-fos* and *c-jun* expression observed (data not shown). Zhou et al. demonstrated that AP-1 dependent gene expression is also induced in myotubes in response to pro-oxidant treatment in a dose-dependent manner and time-dependent manner (34). AP-1 is important mediator of redox-responsive gene expression. The induction of AP-1 continued to increase after 6 h of treatment, peaking between 12 and 48 h (34,35). These results showed that inductions of *c-fos* and *c-jun* expression by paraquat treatment required a time lag. Because paraquat is prototype redox chemical compound, reactive oxygen was generated in the mitochondrial redox reaction after import of paraquat into mitochondria.

The pQBI25 vector has a CMV promoter upstream of the *rsGFP* gene. We hypothesized that *rsGFP* gene expression should be regulated with AP-1 activity because CMV-LTR has TRE for AP-1 binding. In this study, levels of *rsGFP* mRNA and rsGFP protein were induced by paraquat, and a correlation between levels of *c-fos* and *c-jun* mRNA and *rsGFP* mRNA was observed in FR-pQBI25 cells. This result suggests that the induction of rsGFP expression is dependent on induction of c-Fos and c-Jun expression.

The levels of rsGFP protein in FR-pQBI25 cells treated with paraquat were increased by 1.8- to 3.1-fold in comparison with those not treated with paraquat, and the levels of total SOD activity in L2-pRc/CMV-SOD cells treated with paraquat were also increased by 1.4-fold in comparison to those not treated with paraquat. The total SOD activity in the transformed cells is the sum of endogenous rat SOD and exogenous human SOD activities in the pRc/CMV-SOD vector. Considering the rat SOD activity in normal L2 cells (rat lung epithelial-like cell lines), the levels of exogenous human SOD activity in L2-pRc/CMV-SOD cells treated with paraquat were increased approximately twofold in comparison with those of the control conditions. We used two different cells (FR cells as rat skin fibroblasts and L2 cells as rat lung epithelial-like cell lines) in this study. Increase in exogenous gene expression was same intensity in both cell lines. In this fact, we confirmed that effect of induction of exogenous gene expression by treatment of paraquat was not affected by kind of host cell.

To date, heat shock, hormones such as glucocorticoid, estrogen, progesterone and androgen have been used to regulate gene expression. However, for various reasons, including pleiotropic effects caused by the inducers or interference of transgene expression by endogenous inducers, these systems may be not well suited for gene therapy (36). In a previous study, gene expression regulatory systems that used tetracycline-responsive promoters have led to the establishment of highly efficient regulatory systems in mammalian cells (37). Thus, regulation of gene expression during gene therapy is possible.

In this study, paraquat was used to generate reactive oxygen species. Our data suggest that exogenous gene expression driven by the CMV-LTR may be under the regulation of endogenous active oxygen.

If active oxygen is overproduced in tissues and organs, such as in inflammation or in ischemic-reperfusion disorders, exogenous gene expression driven by CMV-LTR should be induced. Since the regulation of exogenous gene expression is possible, a plasmid vector with CMV-LTR is useful in gene therapy for these diseases.

We used rsGFP and SOD as a model reporter in this study. If cDNA encoding proteins with scavenger functions is inserted in the plasmid vector with CMV-LTR, the gene expression should be regulated by the levels of active oxygen, and cytotoxicity resulting from the overproduction of active oxygen in tissues and organs may be reduced.

Some drugs, such as anthracyclines, generate reactive oxygen, which cause adverse effects (38). For prevention of the adverse effects of these drug treatments, a plasmid vector with CMV-LTR may be useful in gene therapy.

CONCLUSION

In the drug delivery system for gene therapy, the regulation of gene expression after gene introduction has been difficult. This study showed that exogenous gene expression driven by CMV-LTR and regulated by AP-1 was induced by active oxygen.

Plasmid vectors with CMV-LTR may be usefulness in gene therapy for medical treatment and prevention of drug-induced side effects of active oxygen intervention and medical treatment of disease that is related to active oxygen.

REFERENCES

1. D. Dreher and A. F. Junod. Role of oxygen free radicals in cancer development. *Eur. J. Cancer* **32A**:30–38 (1996).
2. M. D. Evans, M. Dizdaroglu, and M. S. Cooke. Oxidative DNA damage and disease: induction, repair and significance. *Mutat. Res.* **567**:1–61 (2004).
3. D. Bandyopadhyay, A. Chattopadhyay, G. Ghosh, and A. G. Datta. Oxidative stress-induced ischemic heart disease: protection by antioxidants. *Curr. Med. Chem.* **11**:369–387 (2004).

4. F. M. Veronese, P. Caliceti, O. Schiavon, and M. Sergi. Polyethylene glycol-superoxide dismutase, a conjugate in search of exploitation. *Adv. Drug Deliv. Rev.* **54**:587–606 (2002).
5. B. Young, J. W. Runge, K. S. Waxman, T. Harrington, J. Wilberger, J. P. Muizelaar, A. Boddy, and J. W. Kupiec. Effects of pegorgotein on neurologic outcome of patients with severe head injury. A multicenter, randomized controlled trial. *JAMA* **276**:538–543 (1996).
6. F. Komada, K. Nishiguchi, Y. Tanigawara, S. Iwakawa, and K. Okumura. Effects of secretable SOD delivered by genetically modified cells on xanthine/xanthine oxidase and paraquat-induced cytotoxicity *in vitro*. *Biol. Pharm. Bull.* **22**:846–853 (1999).
7. K. Okumura, K. Nishiguchi, Y. Tanigawara, S. Mori, S. Iwakawa, and F. Komada. Enhanced anti-inflammatory effects of Cu, Zn-superoxide dismutase delivered by genetically modified skin fibroblast *in vitro* and *in vivo*. *Pharm. Res.* **14**:1223–1227 (1997).
8. K. Nishiguchi, F. Komada, Y. Tanigawara, Y. Sakai, S. Iwakawa, and K. Okumura. Effects of Cu, Zn-superoxide dismutase delivered by genetically modified skin fibroblasts on cold-induced skin edema in rats. *Biol. Pharm. Bull.* **12**:1379–1381 (1998).
9. C. A. Mullen, K. Snitzer, K. W. Culver, R. A. Morgan, W. F. Anderson, and R. M. Blaese. Molecular analysis of T lymphocyte-directed gene therapy for adenosine deaminase deficiency: long-term expression *in vivo* of genes introduced with a retroviral vector. *Hum. Gene Ther.* **7**:1123–1129 (1996).
10. B. Avalosse, F. Dupont, and A. Burny. Gene therapy for cancer. *Curr. Opin. Oncol.* **7**:94–100 (1995).
11. T. Inazawa, T. Tanabe, H. Yamada, T. Nakaoka, Y. Hashimoto, T. Yamasaki, H. Kotaki, K. Tani, S. Asano, and N. Yamashita. Glucocorticoid-regulated expression of exogenous human growth hormone gene in rats. *Mol. Ther.* **4**:267–272 (2001).
12. W. Lee, P. Mitchell, and R. Tjian. Purified transcriotional factor AP-1 interacts with TPA inducible elements. *Cell* **49**:741–752 (1987).
13. X. Li and A. Y. Sun. Paraquat induced activation of transcriptional factor AP-1 and apotosis in PC12 cells. *J. Neural. Transm.* **106**:1–21 (1996).
14. T. Curran. FOS and JUN: oncogenic transcriptional factors. *Tohoku J. Exp. Med.* **168**:169–174 (1992).
15. D. Forrest and T. Curran. Crossed signals: oncogenis transcriptional factors. *Curr. Opin. Genet. Dev.* **2**:19–27 (1992).
16. T. Usui, Y. Saitoh, and F. Komada. Induction of CYP3As in HepG2 cells by several drugs. Association between induction of CYP3A4 and expression of glucocorticoid receptor. *Biol. Pharm. Bull.* **26**:510–517 (2003).
17. F. Komada, K. Nishiguchi, Y. Tanigawara, T. Akamatsu, X. Y. Wu, S. Iwakawa, and K. Okumura. Effect of transfection with superoxide dismutase expression plasmid on superoxide anion induced cytotoxicity in cultured rat lung cells. *Biol. Pharm. Bull.* **19**:274–279 (1996).
18. K. Nishiguchi, K. Ishida, M. Nakajima, T. Maeda, F. Komada, S. Iwakawa, Y. Tanigawara, and K. Okumura. Pharmaceutical studies for gene therapy: expression of human Cu, Zn-superoxide dismutase gene transfected by lipofection in rat skin fibroblasts. *Biol. Pharm. Bull.* **19**:1073–1077 (1996).
19. R. Chiu, W. J. Boyle, J. Meek, T. Smeal, T. Hunter, and M. Karin. The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell* **54**:541–552 (1988).
20. T. Hai and T. Curran. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl. Acad. Sci. U. S. A.* **88**:3720–3724 (1991).
21. M. Karin. The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* **270**:16483–16486 (1995).
22. P. Angel and M. Karin. The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. *Biochem. Biophys. Acta.* **1072**:129–157 (1991).
23. P. Angel, M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell* **49**:729–739 (1987).
24. W. Lee, P. Mitchell, and R. Tajian. Purified transcription factor AP-1 interacts with TPA inducible elements. *Cell* **49**:741–752 (1987).
25. M. C. Hollander and A. J. J. Fornace. Induction of fos RNA by DNA-damaging agents. *Cancer Res.* **49**:1687–1692 (1989).
26. M. Buscher, H. J. Rahmsdorf, M. Liftin, M. Karin, and P. Herrlich. Activation of the c-fos gene by UV and phorbor ester: different signal transduction pathway converge to the same enhancer element. *Oncogene* **3**:301–311 (1988).
27. M. Djavaheri-Mergny, J. L. Mergny, F. Bertrand, R. Santus, C. Maziere, L. Dubertret, and J. C. Maziere. Ultraviolet-A induces activation of AP-1 in cultured human keratinocytes. *FEBS Lett.* **384**:92–96 (1996).
28. N. H. Heintz, Y. M. Janssen, and B. T. Mossman. Persistent induction of c-fos and c-jun expression by asbestos. *Proc. Natl. Acad. Sci. U. S. A.* **90**:3299–3303 (1993).
29. A. Puga, D. W. Nebert, and F. Carrier. Dioxin induces expression of c-fos and c-jun proto-oncogenes and large increase in transcriptional factor AP-1. DNA. *Cell Biol.* **11**:269–281 (1992).
30. S. Srinivasan, M. E. Hatley, D. T. Bolick, L. A. Palmer, D. Edelstein, M. Brownlee, and C. C. Hedrick. Hyperglycaemia-induced superoxide production decreases eNOS expression via AP-1 activation in aortic endothelial cells. *Diabetologia* **47**: 1727–1734 (2004).
31. J. Springer, D. Pleimes, F. R. Scholz, and A. Fischer. Substance P mediates AP-1 induction in A549 cells via reactive oxygen species. *Regul. Pept.* **124**:99–103 (2005).
32. M. H. Kim, H. S. Cho, M. Jung, M. H. Hong, S. K. Lee, B. A. Shin, B. W. Ahn, and Y. D. Jung. Extracellular signal-regulated kinase and AP-1 pathways are involved in reactive oxygen species-induced urokinase plasminogen activator receptor expression in human gastric cancer cells. *Int. J. Oncol.* **26**: 1669–1674 (2005).
33. U. Vollgraf, M. Wegner, and C. Richter-Landsberg. Activation of AP-1 and nuclear factor-kappaB transcription factors is involved in hydrogen peroxide-induced apoptotic cell death of oligodendrocytes. *J. Neurochem.* **73**:2501–2509 (1999).
34. L. Z. Zhou, A. P. Johnson, and T. A. Rando. NF kappa B and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells. *Free Radic. Biol. Med.* **31**:1405–1416 (2001).
35. T. Fukushima, K. Yamada, N. Honjo, A. Isobe, K. Shiwaku, and Y. Yamane. Mechanism of cytotoxicity of paraquat. III. The effects of acute paraquat exposure on the electron transport system in rat mitochondria. *Exp. Toxicol. Pathol.* **46**:431–441 (1994).
36. S. Nakagawa and T. Mayumi. Development of novel technology of DDS for gene therapy. *Drug Metab. Pharmacokin.* **18**:223–229 (2003).
37. Z. Zhu, T. Zheng, C. G. Lee, R. J. Homera, and J. A. Elias. Tetracycline-controlled transcriptional regulation systems: advances and application in transgenic animal modeling. *Semin. Cell Dev. Biol.* **13**:121–128 (2002).
38. R. T. Dorr. Cytoprotective agents for anthracyclines. *Semin. Oncol.* **23**:23–34 (1996).