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Regulation of CMV promoter-driven exogenous gene expression with doxorubicin in genetically modified cells

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Abstract

The regulation of gene expression after the introduction of an exogenous gene is a problematic aspect of gene therapy. The purpose of this study was to use doxorubicin to regulate exogenous gene expression in a vector containing the cytomegalovirus (CMV) promoter. The pQBI25 vector, which encodes the CMV promoter and the cDNA for red-shifted green fluorescent protein (rsGFP), was transfected into a rat skin fibroblast cell line (FR cells). The pEGFP vector, encoding the CMV promoter and enhanced green fluorescent protein (EGFP) cDNA, was transfected into human hepatoma HepG2 cells. FR-pQBI25 cells were then continuously exposed to doxorubicin and methotrexate for 96 and 48 h, respectively; HepG2-pEGFP cells were continuously exposed to doxorubicin for 48 h. The levels of *c-fos*, *c-jun* and *rsGFP* mRNA, as well as the levels of rsGFP protein, in the FR-pQBI25 cells were found to be significantly higher following exposure to doxorubicin. However, the level of rsGFP protein was not changed by exposure to methotrexate. The level of EGFP protein in the HepG2-pEGFP cells was also significantly higher following exposure to doxorubicin. To examine the effect of cessation of doxorubicin exposure, FR-pQBI25 cells that had been exposed to doxorubicin for 48 h were re-plated in fresh medium without doxorubicin for a further 48 h. The increased levels of *c-fos, c-jun* and *rsGFP* mRNA and rsGFP protein seen after treatment with doxorubicin had reduced by 48 h after the cessation of exposure to doxorubicin. These findings suggest that CMV-driven exogenous gene expression may be regulated by doxorubicin.

Introduction

The treatment of many types of cancer typically involves the use of anti-cancer agents. Doxorubicin has been used to treat a variety of cancers, including malignant lymphoma, multiple myeloma, acute leukaemia, breast cancer, ovarian cancer, small cell lung cancer and thymic cancer (Ogura 2001). However, doxorubicin also has severe adverse effects, such as myelosuppression, and cardiotoxicity due to the apoptosis of cardiac myocytes (Zhang et al 1996; Ito et al 1999). This raises critical problems in the clinical use of this agent and restricts its full clinical potential (Billingham et al 1978).

Previous ex-vivo studies have assessed the delivery of the superoxide dismutase (SOD) gene into skin fibroblasts and lung epithelial-like cells using a eukaryotic plasmid vector, and the efficacy of cell protection in in-vitro and in-vivo experimental models (Okumura et al 1997; Komada et al 1999). However, at present, gene therapy still has problems in the regulation of gene expression after gene introduction (Harvey & Caskey 1998). Further optimization of gene therapy is required, not only in the induction of gene expression by a regulator but also in the reduction of gene expression by removal/reduction of the regulator.

A eukaryotic expression plasmid vector must encode a promoter region; these promoters are usually sequences from viral long-terminal repeats (LTR). The cytomegalovirus (CMV) promoter is one of the major viral promoters used in eukaryotic expression systems. The CMV promoter is affected by several transcription factors, and the level of gene expression can be regulated in genetically modified cells. In particular, the CMV promoter contains a 12-O-tetradecanoyl phorbol-13-acetate response element (TRE) as an activator protein 1

(AP-1) binding site, and thus AP-1 may affect the level of exogenous gene expression in CMV-promoter-driven expression systems (Lee et al 1987).

The amount of AP-1 is regulated by the levels of *fos* and *jun* mRNAs, and many authors have reported that AP-1 activity is induced by reactive oxygen species (ROS) (Hollander & Fornace 1989; Kim et al 2005; Springer et al 2005). An in-vitro study found that paraquat treatment of rat skin fibroblasts induced the expression of *c-fos* and *c-jun* mRNAs and CMV-driven red-shifted green fluorescent protein (rsGFP) (Kinoshita et al 2006). In addition, a positive correlation was observed between the expression levels of *c-fos* mRNA and *rsGFP* mRNA, most likely because the plasmid vector contained the CMV promoter, which in turn contains a TRE as the AP-1 binding site.

The side-effects of doxorubicin are largely due to the formation of superoxide anions, which arises when doxorubicin is reduced to the semiquinone form at complex I of the mitochondrial electron transport chain, resulting in one-electron transfer to molecular oxygen (Doroshow & Davies 1986). Furthermore, Dorr (1996) reported that the adverse effects induced by doxorubicin cause the generation of ROS from a redox reaction.

The present study examined whether the levels of *c-fos*, *c-jun* and *rsGFP* mRNAs and the level of rsGFP protein change with doxorubicin treatment. The effect of methotrexate treatment on the level of rsGFP protein was also examined. Reduction of CMV-driven rsGFP in transfected cells in-vitro was also examined following cessation of doxorubicin treatment.

Materials and Methods

Materials

Doxorubicin was a kind gift from Kyowahakko Co. (Tokyo, Japan). Methotrexate and Dulbecco's modified Eagle's medium (DMEM) were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Fetal bovine serum (FBS) was purchased from ICN Biomedicals Inc. (Costa Mesa, CA, USA). Penicillin–streptomycin mixture was purchased from Takara Co. (Tokyo, Japan). An RT-PCR kit (One-step RT-PCR kit) was purchased from Qiagen Inc. (Hilden, Germany). *Taq* polymerase and rsGFP- and EGFP-containing eukaryotic expression vectors (pQBI25 and pEGFP, respectively) and recombinant GFP were purchased from Takara Co. (Tokyo, Japan). Geneticin sulfate (G418) was purchased from Invitrogen (Carlsbad, CA, USA.). PCR primers for *c-fos*, *c-jun* and *rsGFP* mRNAs were purchased from Sawady Technology Co., Ltd (Tokyo, Japan); the PCR primer for *β-actin* mRNA was purchased from Funakoshi Co. (Tokyo, Japan). All other chemicals were of reagent grade. All kits were used in accordance with the manufacturer's instructions unless stated otherwise.

Cell cultures and preparation of stable transformants

The rat skin fibroblast cell line (FR cells: ATCC CCL 1213) was obtained from the American Type Culture Collection (Manassas, VA, USA); the human hepatoma cell line (Hep G2 No. RCB0459) was obtained from the RIKEN Cell Bank (Tsukuba, Japan).

FR cells were transfected with pQBI25 using haemaglutinating virus of Japan (HVJ)-liposomes (GenomeONE-Neo. Ishihara Sangyo Co., Osaka, Japan). HepG2 cells were transfected with pEGFP by electroporation using GenePulser (Bio-Rad Laboratories Inc. Hercules, CA, USA). After 48 h, resistant clones were selected in medium containing G418 (final concentration 400 μg mL^{-1}) for 2 weeks. Single clones of each transfected cell were used in this study. FR-pQBI25 cells (FR cells transfected with pQBI25) and HepG2-pEGFP cells (HepG2 cells transfected with pEGFP) were grown in DMEM containing 10% FBS, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (penicillin–streptomycin mixture) at 37°C in a humidified atmosphere of 5% CO_2.

Doxorubicin treatment of FR-pQBI25 cells

The dosage of doxorubicin for patients with cancer is usually 10–75 mg m^{-2} per day. Administration of 50 mg m^{-2} to patients with cancer results in blood concentration of doxorubicin of 0.2–0.3 μM 4 h after administration (Mross et al 1998). The binding rate of doxorubicin to plasma protein is about 80%.

We examined the cytotoxicity of doxorubicin in FR cells using an in-vitro MTT assay. The decrease in cell number was estimated at 20% in test medium containing a final concentration of 0.1 μM doxorubicin compared with control medium. With the same experiment using medium containing 0.5 μM doxorubicin, the decrease in cell number was estimated at 50% compared with control medium. Based on these results, it was considered to be difficult to perform the experiments with more than 0.1 μM doxorubicin, so this concentration was used in the subsequent parts of this study.

Aliquots of 2.2×10^6 FR-pQBI25 cells were seeded into 100 mm tissue culture dishes containing 10% FBS and penicillin–streptomycin mixture at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. The incubation medium was replaced with test medium containing a final concentration of 0.1 μM doxorubicin on day 0. In experiment 1, the test medium was replaced with fresh test medium containing 0.1 μM doxorubicin at 24, 48 and 72 h to study the effect of continuous doxorubicin treatment for 96 h. In experiment 2, to study the effect of cessation of doxorubicin treatment, the test medium was replaced with fresh test medium containing doxorubicin at 24 h; this was then replaced with fresh incubation medium without doxorubicin at 48 h (cessation start, 0 h) and 72 h (24 h after cessation). Cells were harvested at 24, 48, 72 and 96 h after the start of doxorubicin treatment in experiment 1, experiment 2 and control conditions (no doxorubicin).

Doxorubicin treatment of HepG2-pEGFP cells

Aliquots of 1.0×10^6 HepG2-pEGFP cells were seeded into 100 mm tissue culture dishes containing 10% FBS and penicillin–streptomycin mixture at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. The incubation medium was replaced with test medium containing a final concentration of 0.1 μM doxorubicin on day 0. Cells were harvested at 48 h after the start of doxorubicin treatment.

Methotrexate treatment of FR-pQBI25 cells

The effect of methotrexate on rsGFP expression in FR-pQBI25 cells was examined along with the cytotoxicity of methotrexate in FR-pQBI25 cells using an in-vitro WST-1 cell proliferation assay. The IC50 value was estimated as about 200 nM. Thus, 2 and 20 nM methotrexate concentrations were selected for this study.

Aliquots of 1.0×10^6 FR-pQBI25 cells were seeded into 100 mm tissue culture dishes containing 10% FBS and penicillin–streptomycin mixture at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. The incubation medium was replaced with test medium containing a final concentration of 2 or 20 nM methotrexate on day 0. Cells were harvested 48 h after the start of methotrexate treatment.

Quantification of the levels of *rsGFP*, *c-fos* and *c-jun* mRNAs

At 24, 48, 72 and 96 h in both experiment 1 and experiment 2, total RNA was extracted from each culture using a total RNA extraction kit (RNeasy Mini Kit, Qiagen), and the levels of *rsGFP*, *c-fos* and *c-jun* mRNAs were quantified. RT-PCR for *β-actin*, *c-fos, c-jun* and *rsGFP* mRNAs was performed using an RT-PCR kit (Qiagen One-step RT-PCR kit). The resulting agarose gel (2%) was stained with ethidium bromide and visualized on a UV transilluminator. The levels of these mRNAs were quantified from the optical density of their bands on the gels using NIH Image (National Institutes of Health, Bethesda, NJ, USA), and the ratios of the expression levels of *rsGFP*, *c-fos* and *c-jun* mRNAs were normalized relative to that of *β-actin* mRNA. It has previously been shown that the RT-PCR kinetic curves of these ratios of mRNAs do not reach a plateau (Usui et al 2003). In the current study, the RT-PCR kinetic curves of the ratios of the levels of *c-fos*/*β-actin, c-jun*/*β-actin* and *rsGFP* mRNA/*β-actin* mRNAs were determined quantitatively up to a ratio of approximately 3 and the expression of *rsGFP*, *c-fos* and *c-jun* mRNAs quantified.

Determination of rsGFP and EGFP protein levels

Cytosolic proteins were extracted from each culture and the levels of rsGFP and EGFP protein determined. The cells were centrifuged, and the pellet was resuspended in 0.1% Tween solution and left at −80°C for 1 h. The frozen sample was then allowed to stand at room temperature to disrupt the cells, followed by centrifugation at 14 000 rpm for 10 min. The protein levels of rsGFP and EGFP in the supernatant were measured using a fluorometer (excitation wavelength 490 nm; emission wavelength 510 nm) with GFP protein as the standard. Cellular protein levels were determined using a Bio-Rad DC protein assay kit.

Statistical analysis

Statistical analysis was performed using Student's *t*-test or analysis of variance (ANOVA) because the variances of the experimental data (determined using the F-test) were equal in this study. $P < 0.05$ was considered statistically significant.

Results

Time course of *c-fos* and *c-jun* mRNA expression in FR-pQBI25 cells after treatment with or without doxorubicin

In experiment 1, in which FR-pQBI25 cells were continuously exposed to doxorubicin for 96 h, the level of *c-fos* mRNA was significantly higher after 24 h of doxorubicin treatment than in the controls (no doxorubicin). The level of *c-fos* mRNA was increased at 48 h and this level was maintained until 96 h. The level of *c-fos* mRNA was significantly higher than that of the controls at 48, 72 and 96 h (Figure 1).

The level of *c-jun* mRNA was similar to that of the control at 24 h, but was significantly higher at 48 h and remained high until 96 h. The level of *c-jun* mRNA was significantly higher than in the controls at 48, 72 and 96 h (Figure 2).

In experiment 2, we examined the effects of cessation of doxorubicin treatment on the levels of *c-fos* and *c-jun* mRNAs after exposure to the drug for 48 h. The levels of *c-fos* mRNA at 72 h and 96 h (24 and 48 h after cessation) were similar to the levels in experiment 1 after 72 and 96 h of continuous exposure to doxorubicin. However, the levels of *c-fos* mRNA at 72 h and 96 h (24 and 48 h after cessation) in experiment 2 did not significantly differ from the control conditions (no doxorubicin) at 72 and 96 h (Figure 1). The level of *c-jun* mRNA at 72 h (24 h after cessation) was similar to that in experiment 1 at 72 h of continuous exposure to doxorubicin. However, at 96 h (48 h after cessation) the level of *c-jun* mRNA was significantly lower than in

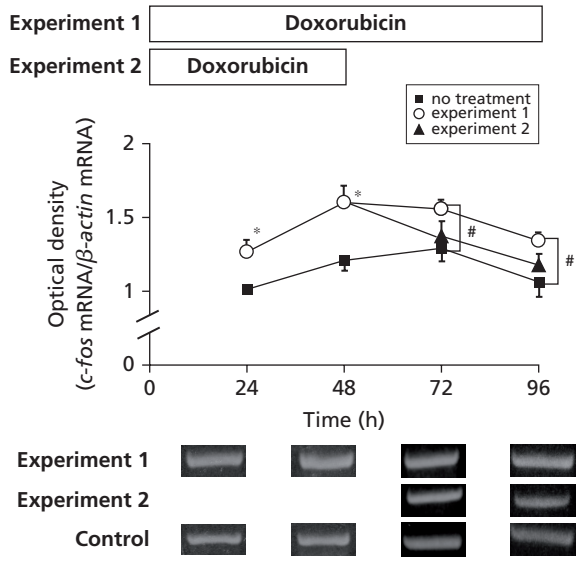

Figure 1 Effects of doxorubicin treatment on *c-fos* mRNA expression in FR-pQBI25 cells. The graph shows the time course of *c-fos* mRNA expression. Each point represents mean ± s.e.m. (n = 4–11). $^*P < 0.05$ (Student's *t*-test). $^\#P < 0.05$ (ANOVA). The lower panel shows representative images of PCR products representing the *c-fos* cDNA in these experimental conditions.

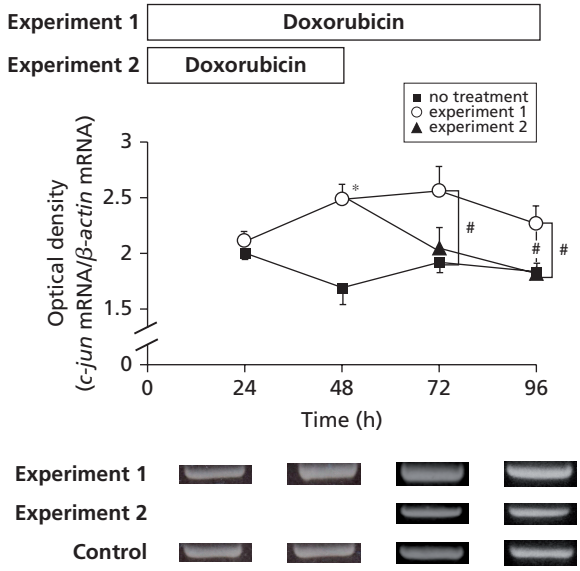

Figure 2 Effects of doxorubicin treatment on *c-jun* mRNA expression in FR-pQBI25 cells. The graph shows the time course of the levels of *c-jun* mRNA expression. Each point represents mean ± s.e.m. (n = 4–11). $^{*}P < 0.05$ (Student's *t*-test). $^{\#}P < 0.05$ (ANOVA). The lower panel shows representative images of PCR products representing *c-jun* cDNA in these experimental conditions.

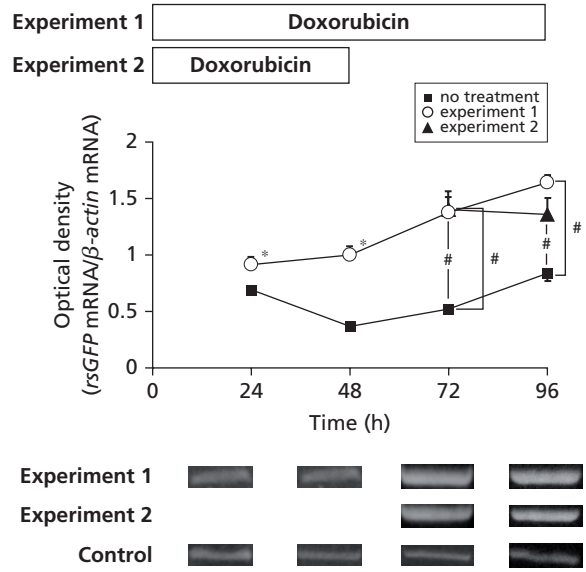

Figure 3 Effects of doxorubicin treatment on *rsGFP* mRNA expression in FR-pQBI25 cells. The graph shows the time course of *rsGFP* mRNA expression. Each point represents mean ± s.e.m. (n = 4–11). $^{*}P < 0.05$ (Student's *t*-test). $^{\#}P < 0.05$ (ANOVA). The lower panel shows representative images of PCR products representing *rsGFP* cDNA in these experimental conditions.

experiment 1 at 96 h of continuous exposure to doxorubicin. No significant difference in the levels of *c-jun* mRNA was observed between experiment 2 at 72 h and 96 h (24 and 48 h after cessation) and the control conditions (no treatment of doxorubicin) at 72 and 96 h (Figure 2).

Time course of *rsGFP* mRNA expression and rsGFP protein expression in FR-pQBI25 cells after treatment with or without doxorubicin

In experiment 1, in which FR-pQBI25 cells were exposed continuously to doxorubicin for 96 h, the levels of *rsGFP* mRNA were significantly higher at 24, 48, 72 and 96 h after doxorubicin treatment compared with the controls (no doxorubicin). In addition, the level of *rsGFP* mRNA increased in a time-dependent manner (Figure 3). The level of rsGFP protein was also significantly higher than in the controls at 24, 48, 72 and 96 h (Figure 4).

Experiment 2 examined the effects of cessation of doxorubicin treatment after drug exposure for 48 h on the levels of *rsGFP* mRNA and rsGFP protein. The levels of *rsGFP* mRNA in experiment 2 at 72 h and 96 h (24 and 48 h after cessation) were the same as those with continuous doxorubicin exposure (experiment 1) at 72 and 96 h. Furthermore, a significant difference was observed in the levels of *rsGFP* mRNA between experiment 2 at 72 h and 96 h (24 and 48 h after cessation) and the control condition at 72 and 96 h (Figure 3). The levels of rsGFP protein in experiment 2 at 72 h and 96 h were significantly lower than at the same time points in experiment 1. A significant difference was also observed in the level of rsGFP protein between experiment 2 at 72 h and 96 h (24 and 48 h after

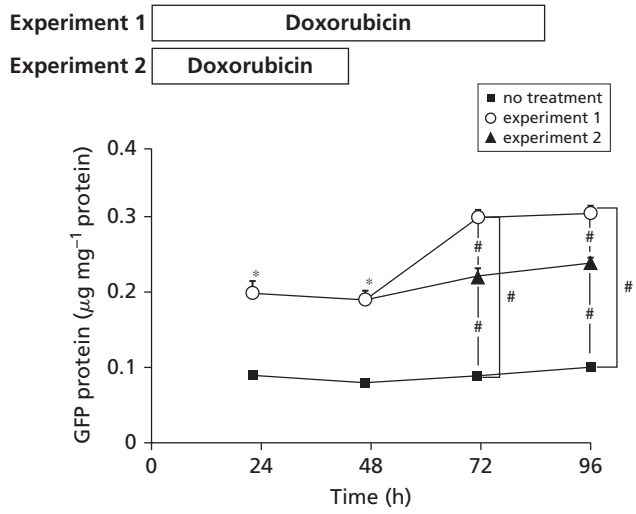

Figure 4 Effects of doxorubicin treatment on rsGFP protein levels in FR-pQBI25 cells. Each point represents mean ± s.e.m. (n = 4–11). $^{*}P < 0.05$ (Student's *t*-test). $^{\#}P < 0.05$ (ANOVA).

exposure) and the control condition (no doxorubicin) at the same time points (Figure 4).

Effects of doxorubicin treatment on EGFP expression in HepG2-pEGFP cells

The levels of EGFP protein were significantly higher at 48 h after doxorubicin treatment compared with the control conditions (no doxorubicin) (Table 1).

Table 1 Effects of doxorubicin treatment on EGFP protein levels in HepG2-pEGFP cells and methotrexate treatment on rsGFP protein levels in FR-pQBI25 cells

	HepG2-pEGFP cells		FR-pQBI25 cells		
Condition	Control	0.1 μM DOX	Control	2 nM MTX	20 nM MTX
GFP (μg mg^{-1} protein)	8.63 ± 0.48	28.70 ± 1.47*	0.0739 ± 0.0011	0.0774 ± 0.0012	0.0808 ± 0.0025

DOX, doxorubicin; MTX, methotrexate.
Values are mean ± s.e.m (n = 4 or 5). *$P < 0.05$ vs control condition in HepG2-pEGFP cells (Student's *t*-test).

Effects of methotrexate treatment on rsGFP expression in FR-pQBI25 cells

The levels of rsGFP protein were not different from control conditions at each concentration of methotrexate (Table 1).

Discussion

This study examined the effect of doxorubicin on CMV-LTR-driven rsGFP expression in transfected rat skin fibroblasts for 96 h in-vitro.

Svensson et al (2007) have reported that chemotherapeutic agents, such as doxorubicin and paclitaxel, induced CMV-promoter-driven exogenous gene expression. They also demonstrated the effects of doxorubicin on p38 mitogen-activated protein kinase. To optimize gene therapy, we need to characterize not only the induction of exogenous gene expression by chemotherapeutic agents but also reduction of exogenous gene expression by cessation of chemotherapeutic agents (i.e. on–off switching of exogenous gene expression by agents).

AP-1 comprises members the FOS and JUN families, and the amount of AP-1 is regulated by the levels of *fos* and *jun* mRNA. The current study demonstrated that the levels of *c-fos* and *c-jun* mRNAs increased during continuous exposure to doxorubicin for 96 h. The level of *c-jun* mRNA in experiment 2 at 96 h (48 h after cessation) was lower than in experiment 1 at 96 h. In addition, the levels of *c-fos* and *c-jun* mRNA in experiment 2 did not differ significantly from the control at 72 and 96 h. A previous study showed that CMV-driven exogenous gene expression was induced by paraquat treatment after 24 h (Kinoshita et al 2006). Both *c-fos* and *c-jun* mRNAs are early response genes and are most often induced within 15–30 min after paraquat exposure (Li & Sun 1996). Gene expression usually returns to the un-induced levels within the first few hours. However, the present data show that gene expression of AP-1 family members can occur later and is sustained for a long period. Zhou et al (2001) reported that AP-1-dependent gene expression is also induced in myotubes in response to pro-oxidant treatment in a dose-dependent and time-dependent manner. We found that *c-fos* and *c-jun* mRNAs were induced by 96 h of continuous doxorubicin treatment. Furthermore, the level of *c-fos* mRNA decreased slowly upon cessation of doxorubicin treatment. However, the level of *c-jun* mRNA decreased more rapidly than *c-fos* mRNA upon cessation of doxorubicin treatment. This difference may be due to the different expression pathways of *c-jun* and *c-fos*. Based on these findings, it is believed that doxorubicin regulates the levels of *c-fos* and *c-jun* mRNA at AP-1 through the generation of ROS.

Levels of *rsGFP* mRNA and rsGFP protein were also induced after 96 h doxorubicin treatment, and the increase in mRNA/protein levels increased with time for 96 h. Cessation of doxorubicin did not significantly reduce the level of *rsGFP* mRNA, although a decreasing trend was observed at 96 h in experiment 2. The level of rsGFP protein after the cessation of doxorubicin at 72 h and 96 h (24 and 48 h after cessation) was significantly lower than that with continuous doxorubicin exposure at 72 and 96 h, respectively. On the basis of these results, it was confirmed that CMV-driven rsGFP gene expression was regulated by doxorubicin treatment.

The effects of doxorubicin treatment on the level of EGFP protein in HepG2-pEGFP cells were also examined, and it was found that the level of EGFP protein was significantly higher (about 3-fold) after 48 h doxorubicin treatment compared with the control conditions; a similar result was seen in FR-pQBI25 cells.

Two different cells lines (FR rat skin fibroblasts and HepG2 human hepatoma cells) were used in this study. The increase in exogenous gene expression showed the same intensity in both cell lines. From this finding it was confirmed that the effect of induction of exogenous gene expression by doxorubicin treatment was not affected by the type of host cell.

Methotrexate, an antifolate drug, shows antineoplastic (antiproliferative) effects resulting from inhibition of dihydrofolate reductase, with a resulting inhibition of purine and pyrimidine synthesis (Cronstein et al 1991). In this study, rsGFP protein was not induced by methotrexate. Therefore, it was considered that induction of exogenous gene expression was not directly associated with the anti-cancer effects of drugs.

Li et al (2007) used an electrophoretic mobility shift assay to study CMV-promoter- and p38-mitogen-activated AP-1. AP-1 is affected by CMV-promoter-driven exogenous gene expression. Kim et al (2007) reported that doxorubicin enhances CMV-driven exogenous gene expression in anaplastic thyroid carcinoma cells. This report demonstrated both induction of CMV-driven exogenous gene expression and induction of nuclear factor (NF)κB. These reports together demonstrate that the CMV promoter not only has a NFκB motif but also a 12-O-tetradecanoyl phorbol-13-acetate (TPA)-response element, which is an AP-1 binding motif. We therefore examined whether AP-1 affected CMV-driven exogenous gene expression. Based on our results, we considered that doxorubicin induces CMV-driven exogenous gene expression by induction of both NFκB and AP-1.

The adverse effects of many anti-cancer drugs are often serious. Doxorubicin causes severe adverse reactions such as neutropenia and cardiotoxicity. Neutropenia is treated by granulocyte colony-stimulating factor (G-CSF) administered by daily injection. If the *G-CSF* gene, instead of the *rsGFP* gene, were inserted into the CMV-promoter-driven plasmid vector, the genetically modified cells would secrete G-CSF protein. Transplantation of these cells into cancer patients who are receiving doxorubicin would result in the expression of G-CSF. We believe that it may be possible to use gene therapy to prevent the adverse effects of anti-cancer agents.

Doxorubicin-mediated cardiotoxicity generates ROS during redox cycling of doxorubicin in cardiac mitochondria (Doroshow & Davies 1986). Thus, the insertion of the genes for several proteins with scavenger functions (e.g. SOD) into the CMV-promoter-driven plasmid vector would mean that gene therapy for the prevention of doxorubicin-induced cardiotoxicity may also be possible.

The ADOC (doxorubicin, cisplatin, vincristine and cyclophosphamide) regimen is used to treat thymic cancer. Doxorubicin and cisplatin are administered on day 1, vincristine on day 3 and cyclophosphamide on day 4. This ADOC therapy is performed for at least two courses at 4-week intervals (Kitami et al 2001). The current findings suggest that exogenous gene expression is induced by doxorubicin exposure and reduced by the cessation of doxorubicin exposure. The elimination half-life of doxorubicin is 25.8 h in humans (Mross et al 1988). If CMV-driven genetically modified cells were transplanted into cancer patients before chemotherapy, exogenous gene expression may be induced by doxorubicin treatment. When the blood concentration of doxorubicin decreases, exogenous gene expression should also decrease. Therefore, the levels of exogenous gene expression could be regulated by doxorubicin concentration, while the optimization of gene therapy for the adverse reactions of doxorubicin would make the system more feasible for use in a clinical setting.

Conclusion

This study examined the effects of doxorubicin in genetically modified cells. The levels of *c-fos* and *c-jun* (AP-1 comprising genes) mRNAs in FR-pQBI25 cells were induced by doxorubicin treatment, and the levels of *c-jun* mRNA were significantly decreased by the cessation of doxorubicin treatment. The levels of *rsGFP* mRNA and rsGFP protein in FR-pQBI25 cells were also induced by doxorubicin treatment; the level of rsGFP protein decreased significantly and level of *rsGFP* mRNA showed a decreasing trend following the cessation of doxorubicin treatment. Furthermore, the level of EGFP protein in HepG2-pEGFP cells was also significantly induced by doxorubicin treatment. In particular, the levels of rsGFP protein in FR-pQBI25 cells were not induced by methotrexate treatment.

On the basis of these results, it is suggested that CMV-promoter-driven exogenous gene expression is regulated by doxorubicin, and that AP-1 is involved in this regulation.

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