JPP
Journal of
Pharmacy and Pharmacology

JPP 2009, 61: 527–531

Received September 24, 2008
Accepted December 12, 2008
DOI 10.1211/jpp/61.03.0016
ISSN 0022-3573

Short Communication

Effects of anticancer agents and scavengers on CMV-promoter-driven exogenous gene expression in genetically modified cells

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Abstract

Objectives This study aimed to investigate whether the levels of *rsGFP* mRNA and the fluorescence levels of cytomegalovirus (CMV)-promoter-driven rsGFP (red-shifted green fluorescent protein) could be changed by using anticancer agents and also to examine the effects of co-treatment with anticancer agents and scavengers.

Methods The pQBI25 vector, which encodes the CMV promoter and the cDNA for rsGFP, was transfected into FR cells (rat skin fibroblast cell line). FR-pQBI25 cells were then exposed to doxorubicin, 5-fluorouracil, methotrexate or paraquat with or without scavengers such as *N*-acetyl cysteine (NAC) and edaravone for 48 h.

Key findings The levels of *rsGFP* mRNA were found to be significantly higher following doxorubicin, 5-fluorouracil and paraquat treatment but were not changed by methotrexate. These levels of *rsGFP* mRNA were found to be significantly lower after paraquat/edaravone co-treatment compared with paraquat alone. The fluorescence levels of rsGFP were found to be significantly higher following doxorubicin and paraquat treatment but were not changed by 5-fluorouracil and methotrexate. The levels were also found to be significantly lower after paraquat/edaravone co-treatment compared with paraquat alone and also after doxorubicin/NAC co-treatment compared with doxorubicin alone.

Conclusions These findings suggest that CMV-promoter-driven exogenous gene expression may be partly regulated by reactive oxygen species.

Keywords anticancer agent; CMV promoter; regulation; scavenger

Introduction

Many peptides and proteins prepared by advanced biotechnology have had applications submitted for clinical use. However, the continuous efficacy and delivery into the target tissue of these drugs are difficult because they are eliminated rapidly from the body. From such a background, gene therapy as a novel drug delivery system was proposed and positive results were obtained in basic and clinical research fields. Nevertheless, at present, gene therapy still has problems in the regulation of gene expression after gene introduction.[1]

A eukaryotic expression plasmid vector must encode a promoter region and these promoters are usually sequences from viral long-terminal repeats. 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 12-*o*-tetradecanoylphorbol-13-acetate response element (TRE) as an activator protein 1 (AP-1) binding site and thus AP-1 may be associated with the level of exogenous gene expression in CMV-promoter-driven expression systems.[2]

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).[3–5] In a previous study, it was found that paraquat treatment of rat skin fibroblasts *in vitro* induced the expression of CMV-promoter-driven red-shifted green fluorescent protein (rsGFP) and *c-fos*, *c-jun* and *rsGFP* mRNAs.[6]

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The treatment of many types of cancer typically involves the use of anticancer agents. However, these agents have severe adverse effects, which raises critical problems in their clinical use and restricts their full clinical potential.[7] Anthracyclines, such as doxorubicin, have severe adverse side effects, including cardiotoxicity and myelosupression, due to the apoptotic cell death of cardiac myocytes.[8,9] Dorr[10] reported that the adverse side effects induced by doxorubicin cause the generation of ROS from a redox reaction. Based on these reports, we previously examined the effects of doxorubicin treatment on the levels of CMV-promoter-driven exogenous gene expression.[11] Consequently, it was found that the levels of *c-fos*, *c-jun* and *rsGFP* mRNAs and the fluorescence level of rsGFP were induced by doxorubicin treatment. Furthermore, it was found that the expression levels of these genes were reduced by the cessation of doxorubicin treatment, suggesting that they were regulated by doxorubicin.

This study examined whether the levels of *rsGFP* mRNA and the fluorescence levels of rsGFP changed upon treatment with several anticancer agents in a rat skin fibroblast cell line. Furthermore, to clarify the cause of the induction of these changes in gene expression, this study also examined the effects of co-treatment with anticancer agents and scavengers such as *N*-acetylcysteine (NAC) and edaravone.

Materials and Methods

Materials

Doxorubicin, methotrexate, 5-fluorouracil, paraquat, NAC, edaravone 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 MP Biomedicals Inc. (OH, US). Penicillin and streptomycin mixture and geneticin sulfate (G418) were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). RT-PCR kit (QIAGEN One Step RT-PCR Kit) was purchased from QIAGEN Inc. (Hilden, Germany). Recombinant GFP was purchased from Takara Co. (Tokyo, Japan). PCR primers for *rsGFP* and *β-actin* mRNAs were purchased from Operon Biotechnologies (Tokyo, Japan). All other chemicals were of reagent grade. All kits were used in accordance with the manufacturer's instructions unless otherwise stated.

Cell cultures and preparation of stable transformants

The rat skin fibroblast cell line (FR cells; ATCC CCL 1213) was obtained from American Type Culture Collection (VA, US). FR cells were transfected with pQBI25 using hemagglutinating virus of Japan (HVJ)-liposomes (GenomeONE-Neo; Ishihara Sangyo Co., Osaka, Japan). After 48 h, resistant clones were selected in medium containing G418 (final concentration 400 μg/ml) for two weeks. Single clones of each transfected cell line were used in this study. FR-pQBI25 cells (FR cells transfected with pQBI25) were grown in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (penicillin–streptomycin mixture) at 37°C in a humidified atmosphere of 5% CO_2.

Treatment of FR-pQBI25 cells with anticancer agents

The cytotoxicity of doxorubicin, methotrexate, 5-fluorouracil and paraquat was examined in FR cells using an MTT assay *in vitro*. The decrease in cell numbers was estimated as approximately 20% in test medium containing a final concentration of 0.1 μM doxorubicin, 20 nM methotrexate, 10 μM 5-fluorouracil or 5 μM paraquat in comparison with control medium. With the same experiment using medium containing higher concentrations of each agent, the decrease in cell numbers was estimated as about 50% in comparison with control medium. Based on these results, it was considered to be difficult to perform experiments with more than 0.1 μM doxorubicin, 20 nM methotrexate, 10 μM 5-fluorouracil or 5 μM paraquat, so these levels were used in the subsequent parts of this study.

Samples of 1.0×10^6 FR-pQBI25 cells were seeded in 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, 20 nM methotrexate, 10 μM 5-fluorouracil or 5 μM paraquat on day 0, respectively. Cells were harvested at 48 h after the start of treatment with anticancer agents and control conditions (no anticancer agents).

Co-treatment of FR-pQBI25 cells with anticancer agents and scavengers

Samples of 1.0×10^6 FR-pQBI25 cells were seeded in 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 mM NAC or edaravone in the described incubation conditions for 2 h and then anticancer agents were added. Cells were harvested at 48 h after the start of treatment with anticancer agents or under control conditions (no anticancer agents).

The effects of NAC and edaravone on the levels of *rsGFP* mRNA and the fluorescence levels of rsGFP were examined in FR-pQBI25 cells. The FR-pQBI25 cells were exposed to 0.1 mM NAC or edaravone for 48 h and the values were estimated as the percentage of the control conditions in each experiment. No significant difference was observed between 0.1 mM NAC/edaravone conditions and the control condition in the levels of *rsGFP* mRNA and the fluorescence level of rsGFP (Table 1). Based on these results, it was decided to use 0.1 mM NAC and edaravone in the subsequent parts of this study.

Table 1 Effect of scavenger treatment on the levels of *rsGFP* mRNA and the fluorescence levels of rsGFP in FR-pQBI25 cells

Condition	*rsGFP* mRNA	rsGFP fluorescence level
Control	100 ± 13.7	100 ± 12.2
0.1 mM NAC	95.7 ± 7.6	82.5 ± 1.8
0.1 mM Edaravone	113.3 ± 10.8	80.2 ± 1.6

NAC, *N*-acetylcysteine. Values estimate percentage of the control in each experiment and represent mean ± SEM, $n = 4–7$.

Quantification of the levels of *rsGFP* mRNA

Total RNA was extracted from each culture using a total RNA extraction kit (RNeasy Mini Kit; Qiagen, Hilden, Germany), and the levels of *rsGFP* mRNA were quantified. RT-PCR for *β-actin* 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 visualised on a lumino image analyser LAS-3000 (FUJIFILM Corporation, Tokyo, Japan). The levels of these mRNAs were quantified using the optical density of their bands on the gels using Multi Gauge (FUJIFILM Corporation, Tokyo, Japan), and the ratios of the expression levels of *rsGFP* mRNA were normalised relative to that of *β-actin* mRNA. It was confirmed previously that the RT-PCR kinetic curves of these ratios of mRNAs did not reach a plateau.[12] The RT-PCR kinetic curves of the ratios of the levels of *rsGFP* mRNA/*β-actin* mRNAs in this study were determined quantitatively at least up to a ratio of approximately 10. Therefore, it was possible to estimate the expression levels of *rsGFP* mRNA quantitatively.

Determination of the fluorescence levels of rsGFP

Cytosolic proteins were extracted from each culture and the fluorescence levels of rsGFP 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 allowed to stand at room temperature to disrupt the cells, followed by centrifugation at 14 000 rev/min for 10 min. The fluorescence intensity in the supernatant was measured using a fluorometer (excitation wavelength 490 nm; emission wavelength 510 nm) with GFP protein as the standard. The levels of total cytosolic protein were also determined using a Bio-Rad DC Protein Assay Kit. The fluorescence levels of rsGFP were normalised by the levels of total cytosolic protein.

Statistical analysis

Statistical analysis was performed using analysis of variance because the variances of the experimental data (determined using the F-test) were equal in this study. $P < 0.01$ was considered statistically significant.

Results

This study examined the effects of anticancer agents and anticancer agent/scavenger co-treatment on the levels of *rsGFP* mRNA and the fluorescence levels of rsGFP in genetically modified cells.

The expression levels of *rsGFP* mRNA and the fluorescence levels of rsGFP in FR-pQBI25 cells were examined (Table 2) and found to be significantly increased by doxorubicin treatment and doxorubicin/scavenger co-treatment compared with the control conditions. Furthermore, the fluorescence levels of rsGFP were significantly lower in the doxorubicin/NAC co-treatment condition compared with doxorubicin treatment alone.

The effect of methotrexate treatment and scavenger co-treatment on levels of *rsGFP* mRNA and the fluorescence levels of rsGFP in FR-pQBI25 cells was examined (Table 3).

Table 2 Effect of doxorubicin and scavenger co-treatment on the levels of *rsGFP* mRNA and the fluorescence levels of rsGFP in FR-pQBI25 cells

Condition	*rsGFP* mRNA	rsGFP fluorescence level
Control	100 ± 13.7	100.0 ± 12.2
0.1 μM Doxorubicin	1163.3 ± 70.8*	186.1 ± 11.1*
0.1 μM Doxorubicin + 0.1 mM NAC	1312.5 ± 32.4*	148.3 ± 7.0*†
0.1 μM Doxorubicin + 0.1 mM edaravone	1242.9 ± 38.4*	211.2 ± 5.3*

NAC, *N*-acetylcysteine. Values estimate percentage of the control in each experiment and represent mean ± SEM, n = 3–7. *$P < 0.01$ vs control; †$P < 0.01$ vs doxorubicin alone.

The levels of *rsGFP* mRNA and the fluorescence levels of rsGFP were not induced by methotrexate treatment or methotrexate/scavenger co-treatment compared with the control conditions.

The effect of 5-fluorouracil treatment and scavenger co-treatment on the expression levels of *rsGFP* mRNA and the fluorescence levels of rsGFP in FR-pQBI25 cells was examined (Table 3). The levels of *rsGFP* mRNA expression were induced significantly by 5-fluorouracil treatment and 5-fluorouracil/scavenger co-treatment compared with the control conditions. However, the fluorescence levels of rsGFP were not induced by 5-fluorouracil treatment or 5-fluorouracil/scavenger co-treatment compared with control condition.

The effect of paraquat treatment and scavenger co-treatment on expression levels of *rsGFP* mRNA and the fluorescence levels of rsGFP in FR-pQBI25 cells was examined and the results are shown in Table 3. The levels

Table 3 Effect of methotrexate, 5-fluorouracil, paraquat and scavenger co-treatment on the levels of *rsGFP* mRNA and the fluorescence levels of rsGFP in FR-pQBI25 cells

Condition	*rsGFP* mRNA	rsGFP fluorescence level
Control	100.0 ± 12.7	100.0 ± 12.2
20 nM Methotrexate	104.6 ± 7.2	87.8 ± 2.7
20 nM Methotrexate + 0.1 mM NAC	121.3 ± 8.3	109.9 ± 13.2
20 nM Methotrexate + 0.1 mM edaravone	126.1 ± 5.5	105.8 ± 2.2
10 μM 5-Fluorouracil	230.2 ± 14.7*	133.2 ± 3.1
10 μM 5-Fluorouracil + 0.1mM NAC	252.5 ± 16.5*	123.7 ± 4.9
10 μM 5-Fluorouracil + 0.1 mM edaravone	221.3 ± 12.1*	125.2 ± 3.1
5 μM Paraquat	1503.2 ± 68.8*	219.5 ± 16.2*
5 μM Paraquat + 0.1 mM NAC	1386.5 ± 59.6*	199.3 ± 4.8*
5 μM Paraquat + 0.1 mM edaravone	1315.6 ± 46.6*†	171.5 ± 7.8*†

NAC, *N*-acetylcysteine. Values estimate percentage of the control in each experiment and represent mean ± SEM, n = 3–7. *$P < 0.01$ vs control; †$P < 0.01$ vs paraquat alone.

of *rsGFP* mRNA and the fluorescence levels of rsGFP were induced significantly by paraquat treatment and paraquat/scavenger co-treatment compared with the control conditions. Furthermore, the levels of *rsGFP* mRNA and the fluorescence levels of rsGFP were significantly lower in the paraquat/edaravone co-treatment conditions compared with paraquat treatment. However, the levels of *rsGFP* mRNA and the fluorescence levels of rsGFP were not reduced between paraquat treatment and paraquat/NAC co-treatment conditions.

Discussion

It is thought that the method of measuring fluorescence intensity indicates functional GFP level effectively. Translational control is a key step in the regulation of gene expression in animal oocytes; mRNA stored in oocytes is masked from the translational machinery by associating with a set of proteins in storage messenger ribonucleoproteins (mRNPs).[13] In addition, mRNA moves between nontranslating and translating mRNP states in the cytoplasm, not only in germ cells but also in cell types as diverse as neurons and yeast.[14,15] We considered that translational control might be changed because the induced *rsGFP* mRNA might be stored in cytoplasm. Alternatively, the stability of mRNA or protein might be changed in cytoplasm. Based on these facts, we think that further investigation into the relationship between mRNA level and protein level, including western blotting analysis, will be performed in the future.

A preceding report showed CMV-promoter-driven exogenous gene expression was induced by paraquat treatment after 24 h.[6] In addition, Svensson *et al.*[16] reported on the effects of chemotherapeutic agents on CMV-promoter-driven exogenous gene expression. They demonstrated the induction of gene expression by chemotherapeutic agents, such as doxorubicin and paclitaxel, and also demonstrated the effects of doxorubicin on p38 mitogen-activated protein kinase. However, to provide optimisation of gene therapy, research should concentrate not only on the induction of the exogenous gene expression system by exposure to chemotherapeutic agents but also on the reduction of exogenous gene expression by cessation of chemotherapeutic agents (i.e. on–off switching of exogenous gene expression system by the agents). Therefore, we previously reported the effects of doxorubicin treatment and cessation of doxorubicin on CMV-promoter-driven exogenous gene expression in genetically modified cells, demonstrating that CMV-promoter-driven exogenous gene expression was induced by doxorubicin treatment and reduced by cessation of doxorubicin treatment.[11] In this study, the expression levels of *rsGFP* mRNA and the fluorescence levels of rsGFP were induced by doxorubicin and paraquat treatment, and the fluorescence levels of rsGFP were significantly reduced by doxorubicin/NAC co-treatment compared with doxorubicin alone. The expression levels of *rsGFP* mRNA and the fluorescence levels of rsGFP were reduced significantly by paraquat/edaravone co-treatment compared with paraquat alone. Therefore, it was considered that the levels of *rsGFP* gene expression were regulated by ROS. However, the levels of *rsGFP* mRNA and the fluorescence levels of rsGFP were not decreased to the basal level by scavenger co-treatment. Kim *et al.*[17] reported that the levels of CMV-promoter-driven exogenous gene expression were induced by doxorubicin in anaplastic thyroid carcinoma cells, and that these effects were mediated by the activation of nuclear factor (NF)-κB in the CMV promoter. Therefore, it was considered that the levels of CMV-promoter-driven exogenous gene expression were regulated not only by AP-1 but also by NF-κB.

The levels of *rsGFP* mRNA were induced by 5-fluorouracil, but the fluorescence levels of rsGFP were not induced by 5-fluorouracil. Wendling *et al.*[18] reported that 5-fluorouracil stimulates the c-Jun/AP-1 signalling pathway, thus it was considered that CMV-promoter-driven exogenous gene expression was induced by 5-fluorouracil treatment.

It was reported previously that the levels of CMV-promoter-driven exogenous gene expression were induced by doxorubicin treatment, and that expression was reduced by cessation of doxorubicin treatment.[11] However, the elimination half-life of doxorubicin in γ-phase is 25.8 h in humans because doxorubicin is readily distributed to peripheral tissues after intravenous administration.[19] Thus, if doxorubicin administration was stopped when the CMV-promoter-driven exogenous gene was overexpressed as an adverse reaction of the agent *in vivo*, the levels of CMV-promoter-driven exogenous gene expression were not reduced rapidly to the basal expression level. Therefore, in these cases, scavenger administration is more effective to reduce the gene expression levels. The mechanism for the anticancer effect of doxorubicin was explained by intercalation to the double-helix structure of DNA. On the other hand, anthracyclines cause irreversible cardiotoxicity, and redox cycling of anthracycline is the most likely.[20] Ikegami *et al.*[21] reported that edaravone prevents anthracycline-induced myocardial cell death. They also examined the effects of daunorubicin/edaravone co-treatment with regard to daunorubicin's anti-tumour effect on human leukaemia K562 cells, and demonstrated that edaravone did not affect daunorubicin's anti-tumour effect on K562 cells.[21] Therefore, we considered that these scavengers do not affect the primary anti-tumour activity of doxorubicin. These findings may contribute to the optimisation of gene therapy for adverse reactions of anticancer agents.

Conclusions

In cancer chemotherapy, adverse reactions occur upon administration of anticancer agents and these adverse reactions are often serious. New strategies for the prevention of adverse reactions to anticancer agents are needed. If CMV-promoter-driven genetically modified cells were transplanted into cancer patients before chemotherapy, exogenous gene expression would be induced by chemotherapy, and the levels of exogenous gene expression could be regulated by the levels of ROS. Thus, the optimisation of gene therapy to reduce adverse reactions due to ROS generated by anticancer agents would make therapy more tolerable for use in a clinical setting. Moreover, if CMV-promoter-driven exogenous genes were regulated for overexpression to occur at the same point as adverse reactions resulting from anticancer agent-generated ROS, the expression levels of these genes may be reduced by scavenger administration.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to declare.

Funding

This work was supported in part by a Grant-in-Aid for Young Scientists (B) (No. 20790152) and Grant-in-Aid for Scientific Research (C) (No. 16590118) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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