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Suppression of Multidrug resistance via Inhibition of Heat Shock Factor by Quercetin in MDR Cells

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Abbreviations: MDR, multidrug resistance; HSF, heat shock factor; HSE, heat shock element; P-gp, P-glycoprotein; FCS, fetal calf serum; GHE-L, golden hamster embryo lung; VCR, vincristine; VBL, vinblastine; CAT, chloramphenicol acetyltransferase

Abstract

***MDR1* promoter has been shown to contain heat shock elements (HSE), and it has been reported that FM3A/M and P388/M MDR cells show a constitutively activated heat shock factor (HSF), suggesting that HSF might be an important target for reversing the multidrug resistance. Therefore, it was examined whether quercetin, which has been shown to interfere with the formation of the complex between HSE and HSF, and to downregulate the level of HSF1, can sensitize MDR cells against anticancer drugs by inhibition of HSF DNA-binding activity. In this study, quercetin appeared to inhibit the constitutive HSF DNA-binding activity and the sodium arsenite-induced HSF DNA-binding activity in the MDR cells. The basal and sodium arsenite-induced MDRCAT activities were remarkably suppressed by the treatment of quercetin. These results were well consistent with the finding that the treatment of quercetin decreased the expression level of P-gp, *MDR1* gene product, in dose-dependent manner, and markedly increased the sensitivity of MDR cells to vincristine or vinblastine. These results suggest that quercetin can decrease the expression of P-gp via inhibition of HSF DNA-binding activity, and might be useful as a chemosensitizer in MDR cells.**

Keywords: multidrug resistance (mdr), heat shock factor (HSF), quercetin

Introduction

The development of resistance of tumor cells to multiple anticancer drugs is one of the critical problems for successful chemotherapy (Beck, 1990; Roninson, 1992; Ling, 1993; Ling, 1997). This resistance can be due to different factors, including failure of drug uptake or activation, alteration in the level of target enzymes such as dihydrofolate reductase and topoisomerase II, activation of enzymatic systems involved in repair of damage to DNA, enhanced expression of detoxifying enzymes such as glutathione S-transferases, and increased anticancer drug efflux (Vendrik *et al*., 1992) This last mechanism of resistance appears to be a major one, as suggested by numerous *in vitro* and clinical studies, and it confers multidrug resistance (MDR) and is usually linked to the overexpression of P-glycoprotein (P-gp), a plasma transmembrane glycoprotein encoded by *MDR1* genes and thought to act as an ATP-dependent drug efflux pump (Chen *et al*., 1990; Pastan and Gottesman, 1991; Gottesman and Pastan, 1993). P-gp gene is normally expressed in human tissues, including liver, kidney, pancreas, and small and large intestine, and P-gp localized at the luminal surface of epithelial cells, suggesting that the pump may have a physiological role in the elimination of xenobiotics or some endogenous metabolites (Thiebaut *et al*., 1997; Fojo *et al*., 1987; Chin *et al*., 1989). However, expression of P-gp in cancer cells is associated with the resistance to several types of antineoplastic drugs, mainly anthracyclines, vinca alkaloids and epipodophyllotoxins (Gottesman and Pastan, 1993).

Although, the mechanism involved in the regulation of *MDR1* gene expression are still unclear, the expression of *MDR1* gene appears to be controlled by various stimuli such as heat shock, heavy metals, differentiation-inducing agents, chemotherapeutics, hormones, and ultraviolet light (Chin *et al*., 1990; Miyazaki *et al*., 1992; Chaudhary and Roninson, 1993; Bates, 1989; Zhao *et al*., 1993). The *MDR1* promoter contains two heat shock elements (HSEs), and there are some evidences that HSEs are required for the response to various stimuli. It has been shown that heat shock or sodium arsenite increases the activity of *MDR1* promoter via HSEs, and Raf-dependent signaling pathway controls the transcription of *MDR1* gene via a mechanism involving the modulation of heat shock factors (HSF) activity (Miyazak *et al*., 1992; Kioka *et al*., 1992; Kim *et al*., 1996).

We have previously demonstrated that MDR cells such as P388/M and FM3A/M cells showed the constitutive HSF DNA-binding activity and the concurrent increase in hsp70 and hsp90 in the absence of stress (Kim *et al*., 1997). The

activation of HSF might be an important transcriptional regulator for inducing *MDR1* gene, and the overexpression of hsp70 confers resistance to at least some anticancer drugs (Kim *et al*., 1996; Kioka *et al*.,1992; Karlseder *et al*., 1996). Therefore, modulation of HSF activity might be a useful target for overcoming MDR. Quercetin (3, 3', 4', 5, 7-pentahydroxyflavone), which is one of the most widely distributed flavonoids in nature, has been shown to interfere with the formation of the complex between the *cis*-acting HSEs in the promoter region of the hsp70 gene and HSF, and to downregulate the level of HSF1 (Hosokawa *et al*., 1992; Nagai *et al*., 1995). Thus, quercetin is a good candidate molecule for downregulation of constitutive HSF DNA binding activity and thereby reversing the MDR phenotype in MDR cells. In the present study, it was examined whether quercetin can sensitize MDR cells against anticancer drugs by inhibition of HSF DNA-binding activity.

Materials and Methods

Materials

P388/M and FM3A/M cells are MDR sublines isolated from parental P388 mouse leukemia cell line and FM3A mouse mammary carcinoma cell line, respectively. The MDR sublines have a classical MDR phenotype with overexpression of the *MDR1* gene as described previously (Kim *et al*., 1993). Both the parental cells and MDR sublines were cultured in RPMI (GIBCO BRL, Gaithersburg, MO) supplemented with 10% fetal calf serum (FCS). Golden hamster embryo lung (GHE-L) cell line (kindly provided by Dr. Yamaguchi Nobuo, Institute of Medical Science, University of Tokyo) was cultured in Dulbecco's modified Eagle's medium (GIBCO BRL, Gaithersburg, MO) supplemented with 10% FCS. The plasmid p432-CAT, which contains a 1 kb genomic fragment containing 0.43 kb region upstream from the major transcription initiation site of human *MDR1* gene linked to bacterial chloramphenicol acetyltransferase (CAT) gene, was obtained from the Japanese Foundation of Cancer Research (Ogura *et al*., 1991). Vincristine, vinblastine, sodium arsenite, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and quercetin were obtained from Sigma (St. Louis, MO). Silica gel plates ($60F_{254}$) were purchased from Merck (Damstadt, Germany). [^{14}C]-Chloramphenicol was purchased from Amersham (Arlington Heights. IL).

Growth inhibition assay and CAT assay

The effect of anticancer drugs on cellular growth in the absence or presence of quercetin was determined using MTT assay as described previously (Mosmann, 1983) The cells were preincubated for 48 h with 20 μM quercetin in 96-well plates. Medium was replaced and various concentrations of vincristine or vinblastine were added. After 48h incubation, MTT was added to each well and plates were incubated in the dark room for 4 h. The water-insoluble MTT-formazan crystals were dissolved in dimethyl sulfoxide, and reduction of MTT was determined at 570 nm using ELISA reader (Bio-Tek Instruments).

To test the effect of quercetin on sodium arsenite-induced *MDR1* promoter activity, GHE-L cells were transfected with 10 μg of p432-MDRCAT according to the calcium phosphate method (Kim *et al*., 1993). After 6 h transfection, cells were subjected to dimethyl sulfoxide shock for 3 min, followed by incubation for additional 40 h. Indicated concentrations of sodium arsenite and/or quercetin then were added to culture medium for the transfected cells, and CAT assay was carried out 8 h later. CAT enzymatic activity in the transfected cells was determined by thin layer chromatography as previously described (Kim *et al*., 1997).

Western blot analysis

The cells were solubilized in 1 × SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and boiled for 4 min. Proteins were electrophoresed in 7.5% poly-acrylamide gel, and transferred to nitrocellulose membrane (Hybond ECL, Amersham Corp., Arlington Heights, IL). The membrane was blocked with 5% non-fat milk, and probed with mouse monoclonal antibody against P-gp (Oncogene Science, Manhasset, NY). Immunoreactive protein was detected using an enhanced chemilumine-scence method (ECL; Amersham Corp.).

Electrophoretic mobility shift assay

For the electrophoretic mobility shift assay, nuclear extracts from cells were prepared as described previously (Kim *et al*., 1997). Cells (2×10^6) were harvested and washed with cold phosphate buffered saline and resus-pended in 50 ml of lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride). The cells were allowed to swell on ice for 10 min, after which the cells were resuspended in 30 ml of lysis buffer containing 0.05% Nonidet P40. Then the tube was vigorously mixed on a vortex machine 3 times for 10 sec, and the homo-genate was centrifuged at 250 *g* for 10 min to pellet the nuclei. The nuclear pellet was resuspended in 40 ml of ice-cold nuclear extraction buffer (5 mM HEPES, pH 7.9, 26% (v/v) glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride), incubated on ice for 30 min with intermittent mixing , and centrifuged at 24,000 *g* for 20 min at 4˚C. The nuclear extract was either used immediately or stored at -70˚C for later use.

Binding reactions were performed in 20 μl mixture containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 2 μg poly (dI-dC), and 10 μg of each cell extract. This mixture was incubated for 10 min at room temperature, and then 0.5 ng of a ^{32}P-labeled oligonucleotide including HSE derived from the human *MDR1* promoter (XbaI-XhoI fragment) was added, and incubation continued for an additional 20 min. After incubation, the reaction mixtures were loaded on 4% polyacrylamide gels and run in 0.5×TBE (44.5 mM Tris-borate, pH 8.0, 1 mM EDTA) at 100 V. Gels were fixed, dried and exposed to X-ray film with an intensifying screen overnight at -70˚C.

Results

Effect of quercetin on constitutive and sodium arsenite-induced HSF DNA-binding activity in MDR cells

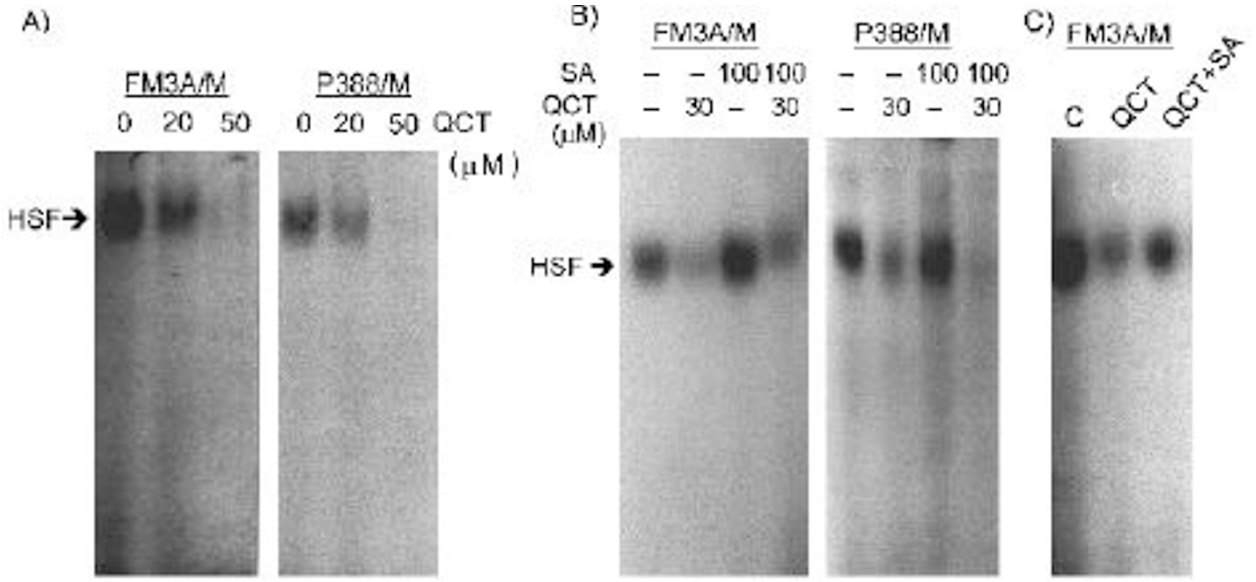
Since FM3A/M and P388/M MDR cells showed a constitutive HSF DNA-binding activity under the non-stressed condition (Kim *et al*., 1997) and quercetin has been shown to interfere with the formation of the complex between the HSE and HSF, and to downregulate the level of HSF1 (Hosokawa *et al.,* 1992; Nagai *et al*., 1995). It was examined whether the constitutive HSF activity in MDR cells could be downregulated by quercetin. When the MDR cells were treated with the indicated concentrations of quercetin, which did not inhibit cell growth, HSF DNA-binding activity was inhibited in a dose-dependent manner in both MDR cells (Figure 1A). These results demonstrated that the constitutively increased HSF DNA-binding activity in MDR cells could be restored by the treatment of quercetin.

It has been previously reported that the transcriptional activation after exposure to arsenite depends on two HSEs in *MDR1* promoter (Kim *et al*., 1996). Therefore, the effect of quercetin on sodium arsenite-induced HSF DNA-binding activity was studied using MDR cells. HSF DNA-binding activity was activated by the treatment of sodium arsenite or was inhibited by the treatment of quercetin, and sodium arsenite-induced HSF DNA-binding activity was inhibited by the treatment of quercetin in both MDR cells (Figure 1B). Pretreatment with quercetin also prevented the ability of sodium arsenite to induce HSF DNA-binding in FM3A/M cells (Figure 1C). These results indicated that quercetin inhibit the induced HSF activity as well as the constitutive HSF activity.

Effect of quercetin on the expression of MDR1 gene in MDR cells

Because quercetin could inhibit the HSF DNA binding activity in MDR cells, it was asked whether the quercetin can suppress the transcription of *MDR1* gene via a mechanism involving the activation of HSF. In this

Figure 1. (A) Effect of quercetin on HSF DNA-binding activity in MDR cell. FM3A/M or P388/M cells were treated with the indicated doses of quercetin (QCT) for 6 h. (B) Effect of quercetin on sodium arsenite-induced HSF DNA-binding activity in MDR cells. FM3A/M or P388/M cells were treated with the indicated doses of sodium arsenite (SA) and/or quercetin (QCT) for 6 h. (C) Effect of the pretreated quercetin on sodium arsenite-induced HSF DNA-binding activity in MDR cells. FM3A/M cells were pretreated with 30 mM quercetin for 6 h, medium was replaced and cells were then treated with 100 mM sodium arsenite for 3 h. Gel mobility-shift analysis of HSF DNA-binding activity was done using a ^{32}P-labeled oligonucleotide including HSE derived from the human *MDR1* promoter (XbaI-XhoI fragment) with the nuclear extracts from each cell.



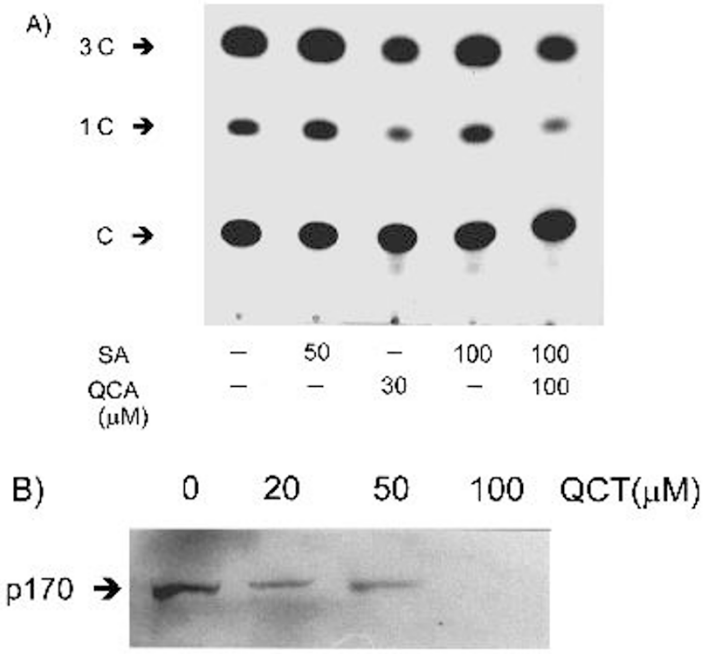


Figure 2. (A) Effect of quercetin on sodium arsenite-induced MDRCAT activity in GHE-L cells. The cells were transiently transfected with 10 μg of p432-CAT. After 24 h of transfection, the cells were treated with the indicated doses of sodium arsenite (SA) and/or quercetin (QCT) for 6 h. The cells were harvested, and aliquots of cell extracts were used to assay for CAT. 3AC, 3-acetyl chloramphenicol; 1AC, 1-acetyl chloramphenicol; C, Chloramphenicol. (B) Effect of quercetin on p170 synthesis in MDR cells. The cells were treated with the indicated doses of quercetin for 6 h, and then whole cell lysates were electrophoresed on 7.5% SDS-polyacrylamide gels and immunoblotted with anti-P-gp (p170) antibody.

Table 1. Effect of quercetin on *in vitro* sensitivities to anticancer drugs of MDR cells.

Cells	Drug	IC_{50}(nM)		
		QCT free	+20 μM QCT pretreatment	Fold Enhancement
FM3A/M	VCR	350	100	3.5
	VBL	660	205	3.2
P388/M	VCR	330	110	3.0
	VBL	850	360	2.4

Cells were treated with anticancer drugs for 48 h after pretreatment of quercetin (QCT) for 48 h.

regard, it was determined whether quercetin inhibit the sodium arsenite-induced MDRCAT activity using GHE-L cells which were transfected transiently with p432-CAT containing various HSF motifs in *MDR1* promoter (Figure 2A). The basal and sodium arsenite-induced MDRCAT activities were remarkably suppressed by the treatment of quercetin and this result was followed by the finding that the P-gp (p170) level was markedly decreased in MDR cells by the treatment of quercetin in dose-dependent manner as shown in western blot analysis (Figure 2B). These results suggested that *MDR1* gene expression could be down-regulated by quercetin treatment via HSF DNA binding to HSEs in MDR promoter, and P-gp level in MDR cells could be restored by the treatment of quercetin.

Effect of quercetin on sensitivity to anticancer drugs of MDR cells

In order to determine whether quercetin can suppress the multidrug resistance, the effect of quercetin on the sensitivity to anticancer drugs of the MDR cells was examined. FM3A/M cells were pretreated for 48 h with quercetin, medium was replaced and cells were then treated with vincristine or vinblastine. After 48 h of incubation, the cytotoxicity was assayed. As shown in Table 1, quercetin pretreatment increased approximately 3-fold the sensitivity of MDR cells to both drugs. These results suggest that quercetin might be useful as a chemosensitizer in MDR cells.

Discussion

There are some evidences that HSF appears to be one of the major regulator of *MDR1* gene expression. Several potential HSEs are located in the *MDR1* promoter region,

and heat shock or sodium arsenite increase the activity of *MDR1* promoter via HSEs (Miyazaki *et al*., 1992; Kim *et al*., 1996; Kioka *et al.*, 1992) . Recently, it has been shown that FM3A/M and P388/M MDR cells isolated in our laboratory have a constitutively activated HSF, and consequently express hsp70, hsp90 and P-gp higher than their parental cells, suggesting that activation of HSF is associated with the increased *MDR1* gene expression and HSF could be an useful target for reversing MDR (Kim *et al*., 1997). Therefore, it is worthwhile to examine whether MDR phenotype could be restored by inhibition of a constitutively active HSF found in MDR cells.

In order to answer this question, MDR cells were treated with quercetin, which is known to inhibit heat shock protein synthesis after heat shock in a human colon carcinoma cell line and Hela cells (Hosokawa *et al*., 1992) and also interfere with the formation of the complex between the HSE and HSF and downregulate the level of HSF1 (Nagai *et al*., 1995). In this study, quercetin appeared to inhibit dose-dependently the constitutive HSF DNA-binding activity and the sodium arsenite-induced HSF DNA-binding activity in the MDR cells. These results indicate that quercetin can inhibit the constitutive activity as well as the induced activity of HSF. Because *MDR1* gene expression was related with the HSF activity, it could be suppressed by the treatment of quercetin. This suggestion was confirmed with the finding that the basal and sodium arsenite-induced MDRCAT activity was inhibited by the treatment of quercetin, and the increased level of P-gp, *MDR1* gene product, was suppressed by the treatment of quercetin in FM3A/M MDR cells.

Since the initial description by Tsuruo *et al*. (1991) that verapamil was able to downmodulate MDR, many compounds were found to inhibit P-gp-mediated transport and, thus, restore active intracellular levels of anticancer drugs and reverse resistance (Ford and Hait, 1990). Such modulators, also termed chemosensitizers, include calcium channel blockers, calmodulin antagonists, quinolines, immunosuppressive drugs, antibiotics, steroids and hormonal analogs, indol alkaloids and surfactants. Most of these compounds have been proposed to function by competing with anticancer drugs for binding sites on P-gp. In addition, there are some evidences that inhibition of protein kinase A downregulates the expression of *MDR1* gene (Kim *et al*., 1993; Abraham *et al*., 1990). In the present study, it was found that quercetin increased markedly the sensitivity of MDR cells to MDR-related drugs, presumably due to the inhibition of constitutive HSF activity. Therefore, quercetin, a bioflavonoid widely distributed in plants, would be able to be used as a novel MDR modulator.

In conclusion, these results suggest that quercetin can suppress the *MDR1* gene expression via inhibition of the HSF DNA-binding activity, and thereby overcome the MDR phenotype.

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