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European Journal of Pharmacology 502 (2004) 195–204

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Selective effects of quercetin on the cell growth and antioxidant defense system in normal versus transformed mouse hepatic cell lines

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Received 8 July 2004; received in revised form 30 August 2004; accepted 1 September 2004
Available online 25 September 2004

Abstract

Quercetin is a dietary anticancer chemical that is capable of inducing apoptosis in tumor cells. However, little is known about its biological effect on nonmalignant cells, although the effect is one of the critical criteria to evaluate the clinical efficacy of the anticancer agent. In this study, we investigated the effects of quercetin on cell growth and apoptosis using embryonic normal hepatic cell line (BNL CL.2) and its SV40-transformed cell line (BNL SV A.8). We also evaluated the effects of quercetin on the antioxidant defense system in those cells. BNL SV A.8 cells were more sensitive to quercetin-mediated cytotoxicity than BNL CL.2 cells. In addition, the enzyme assays showed that quercetin actively stimulated the antioxidant defense systems including superoxide dismutase, catalase, glutathione, and glutathione reductase only in the BNL CL.2 cells. In particular, quercetin significantly reduced superoxide dismutase activity and increased the malonaldehyde content in BNL SV A.8 cells. These are thought to be closely related to quercetin-mediated apoptosis. Our findings suggest that quercetin is a dietary flavonoid that is capable of inducing selective growth inhibition and apoptosis in hepatic tumor cells, but not in normal cells.

Keywords: Quercetin; Hepatocyte; Selective growth inhibition; Apoptosis; Antioxidant defense system

1. Introduction

In recent years, there has been a global trend toward the use of natural bioactive substances as cancer chemopreventive or therapeutic agents (Pezutto, 1997; Christou et al., 2001; Mukherjee et al., 2001). Most of these substances exert their activity by blocking the cell cycle progression and triggering apoptotic cell death. Therefore, cell growth arrest and apoptosis induction in tumor cells has become a prominent indicator of tumor treatment response in employing a plant derived-bioactive substance to reduce and control human mortality due to cancer (Smets, 1994; Paschka et al., 1998). However, development of effective chemopreventive approaches must take into consideration the selective and differential effects manifested by different bioactive substances. Target organ-specific agents that are capable of inducing selective apoptosis of cancer cells, but are harmless to normal cells, are receiving considerable attention in the fields of cancer prevention and therapy (Mukherjee et al., 2001).

Flavonoids are a group of naturally occurring compounds and are commonly found in most plants. They have been recognized as a dietary chemopreventive agent that might block neoplastic inception or delay tumor progression (Gao et al., 1999; Wong and McLean, 1999). In addition, many reports from current studies showed their biological activities affecting basic cell functions, such as proliferation, differentiation, and apoptosis (Formica and Regelson, 1995; Plaumann et al., 1996; Caltagirone et al., 2000). Among the flavonoids, quercetin (3,3′,4′,5,7-pentahydroxyflavone) is one of the most widely studied flavonoids and

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doi:10.1016/j.ejphar.2004.09.012

numerous experiments have reported that quercetin had biological, pharmacological, and medicinal properties (Morel et al., 1993; Hollman and Katan, 1999; Inal and Kahraman, 2000). The beneficial effects of quercetin are thought to be due to the inhibition of enzymes involved in cell cycling and its antioxidant property (Caltagirone et al., 2000; Walker et al., 2000; Aligiannis et al., 2001). Besides the chemopreventive effects, other biological functions of quercetin are believed to improve antioxidant defense systems in living organisms. Quercetin also prevented the ethanol-mediated reduction of intracellular antioxidant defense systems, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione (GSH) (Molina et al., 2003). It was also reported that quercetin reduced ethanol-induced hepatic steatosis and lipid peroxidation (Mizui et al., 1987; Kahraman et al., 2003).

Based on these reports, we hypothesized that quercetin is able to induce selective growth inhibition and apoptosis of tumor cells, and enhance endogenous antioxidant defense systems. In order to confirm this hypothesis, we observed the effects of flavonoid quercetin on normal and transformed mouse hepatocytes, and attempted to ascertain whether the quercetin exhibited tumor cell-specific growth inhibitory or apoptotic activity. We also investigated the effects of quercetin on the antioxidant defense system in cells in order to observe the mechanism inherent in the induction of apoptosis in transformed cells.

2. Materials and methods

2.1. Chemicals and laboratory wares

Unless otherwise specified, all chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) and all the laboratory wares were from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA). Quercetin was obtained from Sigma and dissolved in dimethylsulfoxide (DMSO) prior to use. The final concentration of DMSO did not exceed 0.1% (v/v) throughout the experiments.

2.2. Cell culture and treatment

Embryonic normal hepatic cell line, BNL CL.2 cells, and its SV40-transformed cell line, BNL SV A.8 cells, which were found as negative for ectromelia virus (mousepox) (Patek et al., 1978), were cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA). Especially, BNL SV A.8, but not BNL CL.2 cells were known to be tumorigenic in immunosuppressed mice and need more fluid renewal than BNL CL.2 cells in culture. Cultures were switched to a fresh batch of the same medium before quercetin treatment was added. At various times after the treatment, cells were processed for measuring their cell growth, apoptosis, activity of intracellular antioxidant systems, and malonaldehyde content.

2.3. Determination of DNA synthesis

The level of DNA synthesis by the normal or transformed hepatocytes after quercetin treatment was measured by adding 1 μCi of [methyl-^{3}H] Thymidine (Amersham Pharmacia Biotech, Piscataway, NJ, USA) to each well of 96-well culture plates for the last 12 h of various culture periods. The cells were then collected with a cell harvester (Inotech, Switzerland), and the tritium contents were measured using a liquid scintillation counter (Packard Instrument, Downers Grove, IL).

2.4. Determination of cytotoxicity

Cellular cytotoxicity induced by quercetin treatment was measured using a trypan blue exclusion assay. Briefly, the hepatic cells were cultured in DMEM and supplemented with 10% fetal bovine serum in the presence of 1–200 μM quercetin for various time periods. After incubation, the cells were stained with 0.4% trypan blue and about 100 cells were counted for each treatment. Cytotoxicity was calculated as follows: % cytotoxicity=[(total cells−viable cells)/total cells]×100.

2.5. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay

After exposure to quercetin various times, the hepatic cells were fixed with 1% buffered formaldehyde (pH 7.5) for 30 min while on ice. The cells were then washed with PBS, resuspended in 70% ice-cold ethanol, and kept at −20 °C for 1 h. The cells were rehydrated with PBS and incubated in a TdT buffer containing 30 mM Tris–HCl (pH 7.2), 140 mM sodium cacodylate, 1 mM $CoCl_2$, 0.05 mg/ml bovine serum albumin, 0.1 mM dithiothreitol, 7.5 U/ml TdT, and 0.4 nM/ml FITC-5-dUTP. After a 30-min incubation at 37 °C, the reaction was blocked by transferring the cells to a buffer containing 300 mM sodium chloride, 30 mM sodium citrate, and 2% bovine serum albumin for 30 min. Finally, the cells were washed with PBS and observed under a fluorescence microscope (Axioskop 2, Carl Zeiss, Germany).

2.6. DNA fragmentation assay

After quercetin treatment, the hepatic cells were incubated with a lysis buffer (1% NP-40 and 1% sodium dodecyl sulfate (SDS) in 50 mM Tris–HCl, pH 8.0) for 1 h at 65 °C. DNA was extracted with phenol/chloroform/isoamyl alcohol and the degree of fragmentation was analyzed using a 2% agarose gel electrophoresis followed by an ethidium bromide staining.

2.7. Cell cycle analysis

Quercetin-induced DNA fragmentation was also determined by a flow cytometric analysis after propidium iodide staining. Initially, the suspension (2×10^6 cells) of quercetin-treated cells was fixed for 24 h at 4 °C with 80% ethanol and then they were incubated overnight at 4 °C with 1 ml of propidium iodide staining mixture (250 μl of PBS, 250 μl of 1 mg/ml RNase in 1.12% sodium citrate, and 500 μl of 50 μg/ml propidium iodide in 1.12% sodium citrate). After the staining, 1×10^4 cells were analyzed with the FACS Calibur® system (Becton Dickinson, San Jose, CA).

2.8. Enzyme assays

At the varied times of quercetin treatment, the hepatic cells were rinsed in ice-cold PBS and destroyed by repeated freezing and thawing in a 50 mM phosphate buffer (pH 7.8) containing 0.1% Triton X-100 and 0.1 mM EDTA. The supernatants were then centrifuged at $15,000\times g$ at 4°C for 20 min, and used as hepatic extract. Protein concentration was determined by the method of Bradford (1976).

Total SOD activity was determined according to the method of Misra and Fridovich (1972). Enzyme activity was expressed as U/mg protein and calculated from a standard SOD curve. Manganese SOD (MnSOD) was determined by adding 100 μl of 20 mM sodium cyanide to inhibit copper–zinc SOD (CuZnSOD) activity. CAT activity was determined by the method of Aebi (1984) with a slight modification. The hepatic extract (10 μl) was briefly mixed with 240 μl of a phosphate buffer in a cuvette before adding 250 μl of 66 mM H_2O_2 that was diluted to the phosphate buffer. The decomposition of H_2O_2 was measured at 240 nm for 1 min. Enzyme activity was expressed as U/mg protein and calculated from a standard CAT curve. GPX activity was determined by a modified version of Flohe and Gunzler (1984). Fifty microliters of the hepatic extract was added into the reaction mixture that consisted of 550 μl of phosphate buffer, 100 μl of 10 mM GSH, 100 μl of 1.5 mM NADPH, and 100 μl of 0.24 U GR. It was then incubated at 37 °C for 10 min. After that, 100 μl of 12 mM *tert*-butyl hydroperoxide was added to the reaction mixture and then the decrease of NADPH concentration was monitored at 340 nm for 3 min. The GPX activity was expressed as nmol of NADPH/min/mg protein. For the determination of GR activity, 50 μl of 2 mM NADPH in 10 mM Tris buffer (pH 7.0) was added in a cuvette containing 50 μl of 20 mM oxidized GSH (GSSG), in phosphate buffer containing 0.1 mM EDTA (pH 7.0) and 850 μl of phosphate buffer. After that, 50 μl of the hepatic extract was added to the NADPH–GSSG buffered solution and measured at 340 nm for 3 min. The GR activity was determined by measuring the decrease of NADPH concentration and expressed as nmol of NADPH/min/mg protein. Finally, GSH was assayed in the hepatic cells according to Dringen and Hamprecht method (1996). The total number of intracellular GSH was determined from a standard GSH curve and then expressed as μmol/mg protein.

2.9. Lipid peroxidation assay

This assay is used to determine the intracellular malonaldehyde level as described by Ohkawa et al. (1979). Here, 100 μl of the hepatic extract was briefly mixed with 50 μl of 8% SDS and incubated for 10 min at room temperature. The mixtures were resuspended in a buffer containing 375 μl of 20% acetic acid (pH 3.5) and 375 μl of 0.6% thiobarbituric acid, and incubated at 80 °C for 60 min. After that, 250 μl of distilled water and 1.25 ml of butanol:pyridine mixture (15:1, v/v) was added to the mixtures and centrifuged at $1000\times g$ for 5 min. Malonaldehyde levels in the supernatants were measured at 532 nm and expressed as μmol/mg protein using 1,1,3,3-tetraethoxypropane as a standard.

2.10. Measurement of reactive oxygen species

Intracellular reactive oxygen species were detected using flow cytometric analysis as described by Bass et al. (1983). Briefly, a stock solution of 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) (50 mM; Calbiochem, Darmstadt, Germany) was prepared in DMSO and stored at −20 °C in the dark. Cells (10^7 cells/ml) were exposed to 100 μM of quercetin for various times and subsequently incubated with 25 μM DCFH-DA for 20 min. The green fluorescence of DCF was recorded at 515 nm (FL 1) using a FACS Calibur® system (Becton Dickinson), and 10,000 events were counted per sample.

2.11. Statistical analyses

All data are expressed as mean±standard error (S.E.). A one-way analysis of variance using SPSS ver. 10.0 software was used for multiple comparisons. A value of $P<0.05$ was considered significant.

3. Results

3.1. Effects of quercetin on proliferation of the normal and transformed hepatic cells

The effect of quercetin on cell proliferation was determined by tritium incorporation using two types of hepatic cells; BNL CL.2 and BNL SV A.8 cells, which all originated from the same strain of mouse at the same developmental stage. As shown in Fig. 1A, addition of quercetin to the cultured BNL CL.2 cells resulted in a slight inhibition of tritium incorporation by the DNA of the cells. In contrast, quercetin treatment to BNL SV A.8 cells induced a dramatic inhibition of tritium uptake by the cells. When cells were treated with 100 μM quercetin for 24 h,

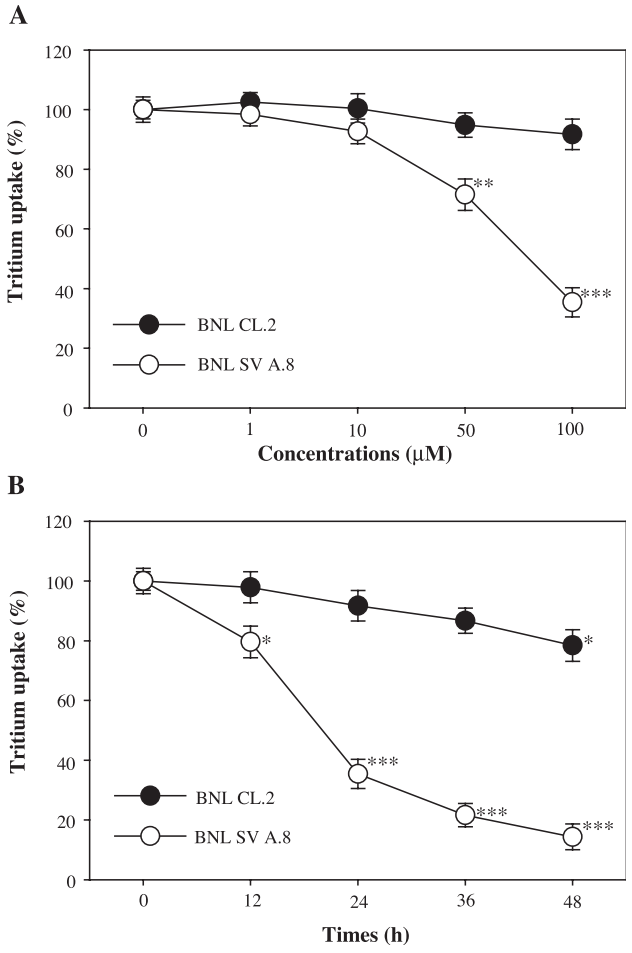

Fig. 1. Effects of quercetin on DNA synthesis in hepatic cells. (A) BNL CL.2 and BNL SV A.8 cells were treated with the indicated doses of quercetin for 24 h and then incubated for another 12 h in the presence of [methyl-^{3}H] TdR. (B) Cells were treated with 100 μM of quercetin for the indicated times and incubated with [methyl-^{3}H] TdR for the last 12 h of the incubation period. The results represent the mean±S.E. of experiments performed in triplicate. *P<0.05, **P<0.01, and ***P<0.001 represent significant differences between the experimental and control value.

tritium uptake decreased to 35.4% of the normal level for the untreated cells. In addition, the quercetin-mediated inhibition of DNA synthesis in BNL SV A.8 cells were time-dependent (Fig. 1B). These results indicated that transformed BNL SV A.8 cells are more sensitive to quercetin-mediated inhibition of proliferation than the BNL CL.2 cells are. The low-dosage and short duration quercetin treatment has a significantly greater inhibitory effect on the proliferation of BNL SV A.8 cells, than on the proliferation of BNL CL.2 cells.

3.2. Effects of quercetin on cytotoxicity of normal and transformed hepatic cells

To determine how quercetin inhibited hepatic cell proliferation, we attempted to ascertain whether quercetin exerted a cytotoxic effect on the hepatic cells. We did this by monitoring trypan blue exclusion after the quercetin treatment (Fig. 2). The added quercetin did not exert a cytotoxic effect in BNL CL.2 cells and the cytotoxicity induced by a 24-h incubation of cells with 100 μM quercetin was only 6.4% (Fig. 2A). However, more than 13% of BNL SV A.8 cells stained positively with trypan blue when they were treated with 100 μM quercetin for 24 h. A time-course study revealed that BNL SV A.8 cells were more sensitive to quercetin-mediated cytotoxicity than the BNL CL.2 cells were (Fig. 2B).

3.3. Quercetin-mediated apoptosis in normal and transformed hepatic cells

To further understand the effect of quercetin on hepatic cells, the quercetin-treated hepatocytes were subjected to apoptosis assays, including TUNEL assay, agarose gel electrophoresis of genomic DNA, and cell cycle analysis after propidium iodide staining (Fig. 3; Tables 1 and 2). Initially, a dose-dependent increase in the number of positively stained hepatic cells after quercetin treatment

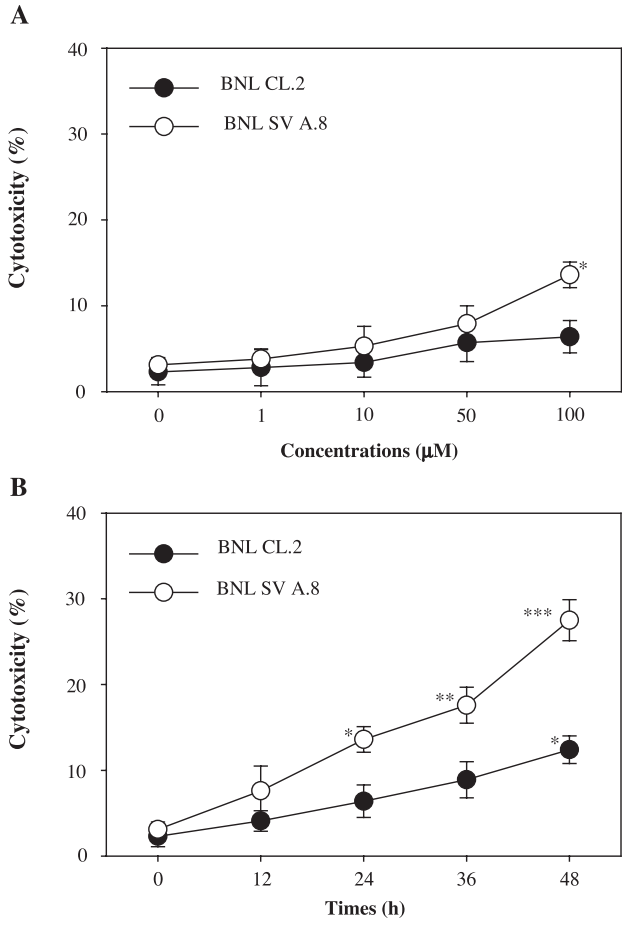

Fig. 2. Cytotoxic effects of quercetin in hepatic cells. BNL CL.2 and BNL SV A.8 cells were treated with the indicated doses of quercetin for 24 h (A) or with 100 μM of quercetin for the indicated times (B), and then processed for trypan blue staining. Results represent the mean±S.E. of three separate experiments. *P<0.05, **P<0.01, and ***P<0.001 represent significant differences between the experimental and control value.

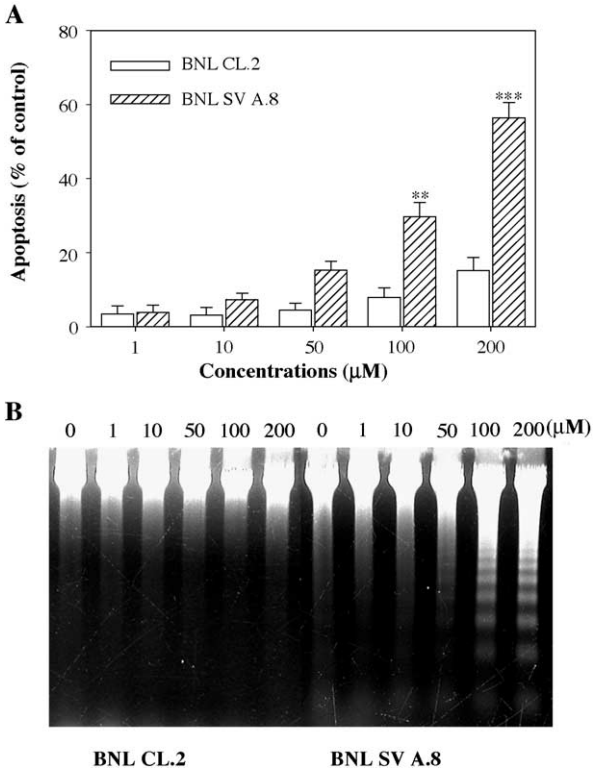

Fig. 3. Quercetin-mediated apoptosis in hepatic cell lines. BNL CL.2 and BNL SV A.8 cells were incubated in the presence of 1–200 μM of quercetin for 24 h. (A) The cells were stained with FITC-conjugated dUTP and the degree of apoptosis was assessed. Each bar shows the mean±S.E. of three separate experiments and $^{**}P<0.01$ and $^{***}P<0.001$ represent significant differences between the experimental and control value. (B) Genomic DNA was prepared and analyzed by 2% agarose gel electrophoresis followed by ethidium bromide staining. A representative result from three independent experiments is shown.

was observed in BNL SV A.8 cells by the TUNEL assay (Fig. 3A). After 24 h of exposure to 100 or 200 μM quercetin, 29.7% and 56.4% of BNL SV A.8 cells, respectively, were observed to be apoptotic. In contrast, BNL CL.2 cells were relatively resistant to quercetin-mediated apoptosis and only 15.1% of the cells were

Table 1
Effect of quercetin on the cell cycle distribution of BNL CL.2 cells

Concentrations (μM)	% Cell			
	Sub-G_0/G_1	G_0/G_1	S	G_2/M
0	3.3±0.6	56.2±3.1	12.4±1.6	28.1±1.5
1	3.1±0.8	54.6±3.3	12.6±1.6	29.7±2.2
10	3.4±0.3	52.1±4.1	11.6±1.4	32.9±2.3
50	4.1±0.7	54.3±3.8	12.9±2.4	28.7±1.6
100	5.7±0.5	53.1±2.9	14.1±1.6	27.1±1.2
200	30.8±2.7[a]	28.2±2.2[a]	20.2±1.9	20.8±2.2

BNL CL.2 cells were treated with the indicated doses of quercetin for 48 h. The percentage of cells in each phase of the cell cycle was calculated through WinMDI 2.8 program of propidium iodide fluorescence data. Data represent the mean±S.E. of experiments performed in triplicate.

[a] $P<0.001$ represents a significant difference between the experimental and control value.

Table 2
Effect of quercetin on the cell cycle distribution of BNL SV A.8 cells

Concentrations (μM)	% Cell			
	Sub-G_0/G_1	G_0/G_1	S	G_2/M
0	3.5±0.9	63.1±3.1	11.8±1.6	21.6±1.6
1	3.0±1.1	64.3±3.8	12.3±1.9	20.4±1.4
10	2.9±0.6	62.2±5.2	11.7±1.6	23.2±1.8
50	15.6±2.8[a]	38.8±2.2[b]	14.2±1.3	31.4±1.9[c]
100	79.3±5.5[b]	10.0±1.1[b]	2.6±1.2[c]	8.1±1.8[a]
200	89.3±6.7[b]	5.7±1.2[b]	1.3±0.1[a]	3.7±1.6[b]

BNL SV A.8 cells were treated with the indicated doses of quercetin for 48 h. The percentage of cells in each phase of the cell cycle was calculated through WinMDI 2.8 program of propidium iodide fluorescence data. Data represent the mean±S.E. of experiments performed in triplicate.

[a] $P<0.01$ represent significant differences between the experimental and control value.

[b] $P<0.001$ represent significant differences between the experimental and control value.

[c] $P<0.05$ represent significant differences between the experimental and control value.

apoptotic, even when they were treated with 200 μM quercetin for 24 h. The induction of quercetin-mediated apoptosis was further examined by investigating the DNA ladder formation after quercetin treatment (Fig. 3B). As shown in the figure, the quercetin treatment induced dose-dependent apoptotic ladder formation of genomic DNA in BNL SV A.8 cells. This formation of DNA ladders were observed after the quercetin treatment was as low as the dosage of 50 μM. Quercetin did not, however, induce a prominent DNA laddering in BNL CL.2 cells, even at the very high concentration level (200 μM quercetin). Differences in sensitivity to quercetin-mediated apoptosis between normal and transformed hepatic cells were further confirmed through propidium iodide staining (Tables 1 and 2). Following the treatment with 50 or 100 μM quercetin for 48 h, 15.6% and 79.3% of BNL SV A.8 cells became apoptotic, respectively (Table 2). Meanwhile, only 30.8% of BNL CL.2 cells were apoptotic, even when the cells were treated with 200 μM quercetin for 48 h (Table 1). These results indicated that transformed BNL SV A.8 cells are much more sensitive to quercetin-mediated apoptosis than that of the normal hepatic BNL CL.2 cells.

3.4. Effects of quercetin on the activity of intracellular antioxidant systems

To determine whether quercetin alters the antioxidant defense systems of the hepatic cells, we carried out enzyme assays for various intracellular antioxidant enzymes (Figs. 4–6). Initially, quercetin treatment increased the intracellular SOD activity in BNL CL.2 cells (Fig. 5). After the treatment with 100 μM quercetin for 24 h, the total SOD level in the BNL CL.2 cells increased by a factor of 1.46 from the level of untreated cells (6.75 U/mg protein) ($P<0.05$). This quercetin-mediated increase in SOD activity was not maintained but it did significantly decrease in the BNL

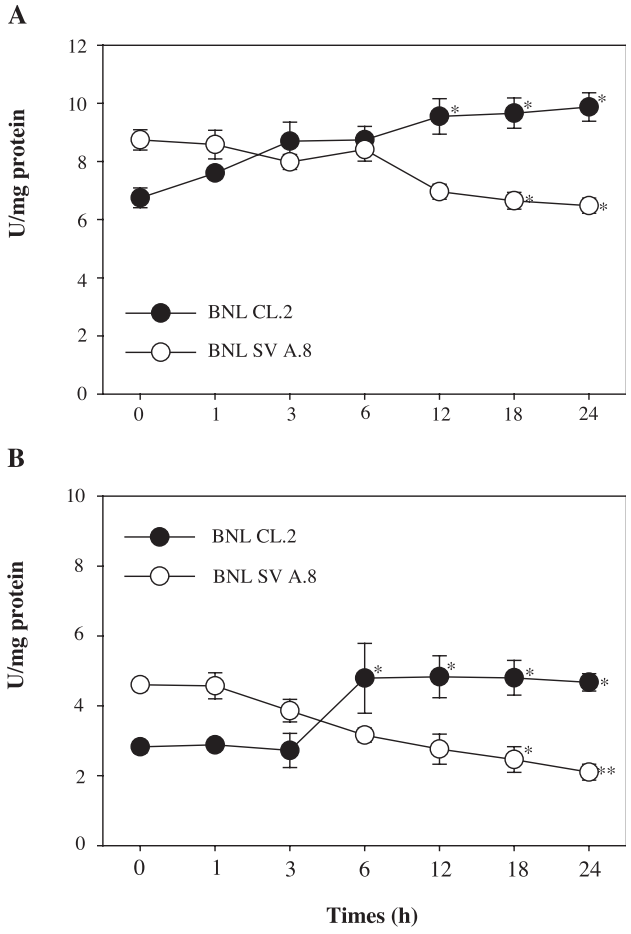

Fig. 4. Superoxide dismutase (SOD) activity in hepatic cells treated with quercetin. BNL CL.2 and BNL SV A.8 cells were treated with 100 μM of quercetin over various times of (0–24 h). After incubation, total SOD (A) and MnSOD activity (B) in the cells were determined and described in Materials and methods. The figure shows a representative result from triplicates and expressed as the mean±S.E. *P<0.05 and **P<0.01 represent significant differences between the experimental and control value.

SV A.8 cells. In order to determine whether the quercetin-induced increase in the total SOD activity induced by quercetin treatment was due to the elevation of MnSOD levels, we subsequently measured the MnSOD activity (Fig. 4B). The results showed that the quercetin-induced increase of total SOD activity in BNL CL.2 cells were, indeed, due specifically to the increased MnSOD activity. When 100 μM quercetin was added to BNL CL.2 cells for 24 h, MnSOD activity was increased by 1.66-fold (P<0.05), compared to that of untreated cells (2.82±0.17 U/mg protein). Whereas 54.6% reduction of MnSOD activity in BNL SV A.8 cells was observed after the same treatment.

A similar pattern of quercetin-mediated selective increase in intracellular CAT activity was observed in BNL CL.2 cells (Fig. 5). As shown in the figure, when the BNL CL.2 cells were treated with 100 μM quercetin at various times, a significant increase in CAT activity started from the 6th hour of incubation and at 24th hour of incubation the CAT activity was augmented by 2.16-fold (P<0.01) in BNL CL.2 cells, when compared with the control value (3.12 nmol/min/ml). In contrast, the enhancement in CAT activity was not observed in BNL SV A.8 cells, even though it had the same treatment of quercetin. The time-course study of intracellular GSH content in hepatic cells was also performed (Fig. 6A). As shown in the figure, there was a significant increase in the GSH content in BNL CL.2 cells. When BNL CL.2 cells were treated with 100 μM quercetin for 24 h, the intracellular GSH concentration increased 2.05-fold higher (P<0.05) than that of the untreated cells (2.53 μmol/mg protein). In GR activity, quercetin treatment to the hepatic cells also caused a significant increase only in BNL CL.2 cells in a time-dependent manner (Fig. 6B). When BNL CL.2 cells were treated with 100 μM, GR activity was augmented by 1.59-fold (P<0.05) at 6 h and 1.81-fold (P<0.01) at 12 h incubation of the treatment, compared to that of untreated cells (27.4 nmol/min/ml). However, GPX activity was not affected by the quercetin treatment in both the cells (Fig. 6C). These results suggest that quercetin has a selective influence on intracellular antioxidant systems. It has shown that quercetin increases intracellular activities of SOD, CAT, and GR, and the GSH concentrations in BNL CL.2 cells, but not in the transformed BNL SV A.8 cells.

3.5. Effect of quercetin on lipid peroxidation in normal and transformed cells

Subsequently, we examined the effect of quercetin on lipid peroxidation using the hepatic cells (Fig. 7). As shown in the figure, there was a significant increase of malonaldehyde content in BNL SV A.8 cells. When BNL SV A.8 cells were treated with 100 μM of quercetin, the level of malonaldehyde content was increased by 2.74-fold

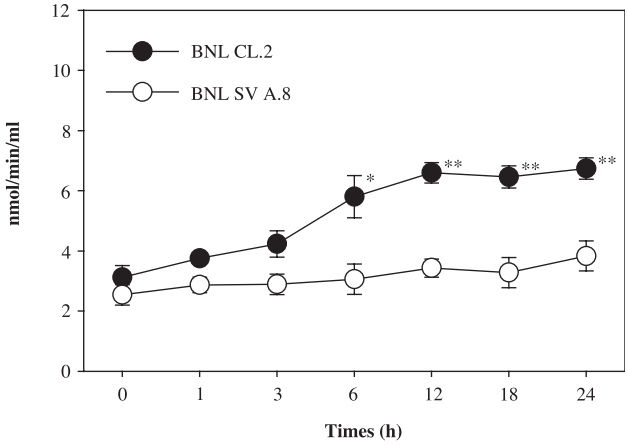

Fig. 5. Catalase (CAT) activity in hepatic cells treated with quercetin. BNL CL.2 and BNL SV A.8 cells were treated with 100 μM of quercetin over various times of (0–24 h). After incubation, CAT activity was determined as previously described. The figure shows a representative result from triplicates and expressed as the mean±S.E. *P<0.05 and **P<0.01 represent significant differences between the experimental and control value.

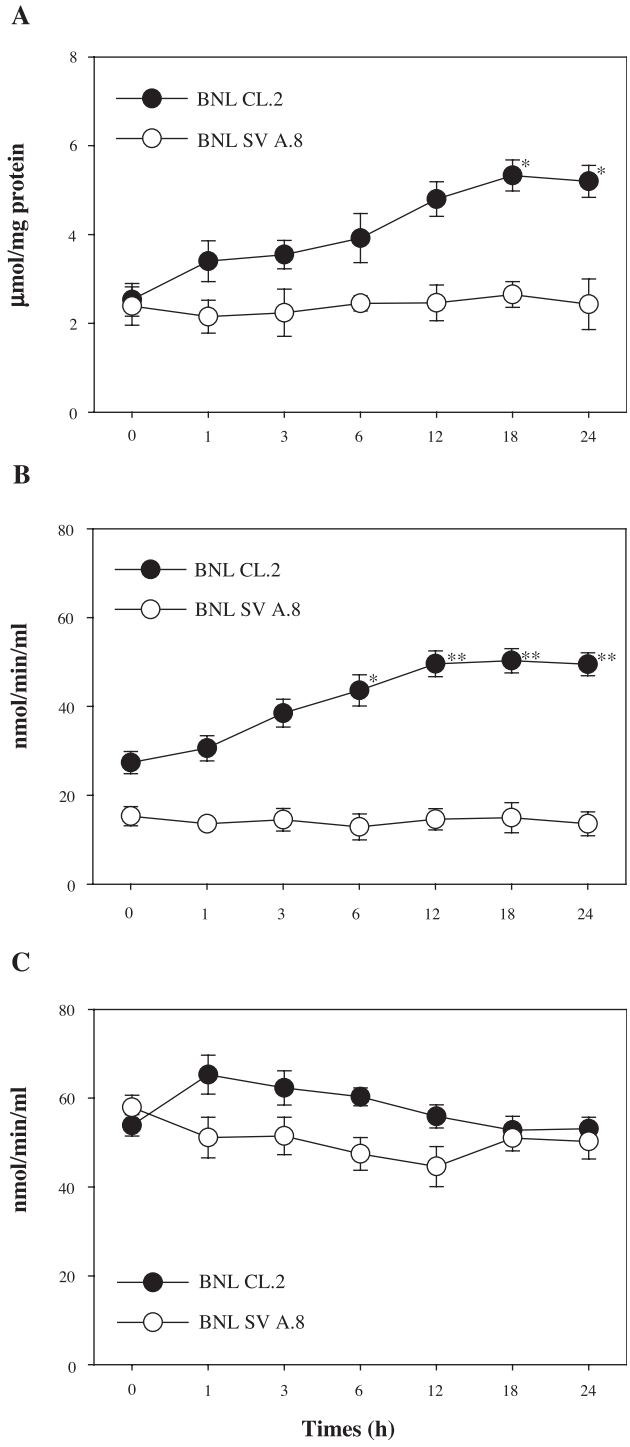

Fig. 6. The activities of thiol compound-related antioxidants in hepatic cells treated with quercetin. BNL CL.2 and BNL SV A.8 cells were treated with 100 μM of quercetin over various times of (0–24 h). After incubation, intracellular glutathione (GSH) content (A) and glutathione reductase (GR) (B) and glutathione peroxidase (GPX) activities (C) in the cells were determined as previously described. Each figure shows a representative result from triplicates and expressed as the mean±S.E. *P<0.05 and **P<0.01 represent significant differences between the experimental and control value.

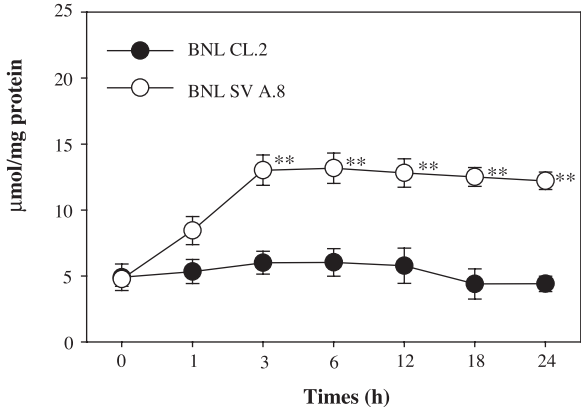

Fig. 7. Malondialdehyde level in hepatic cells treated with quercetin. BNL CL.2 and BNL SV A.8 cells were treated with 100 μM of quercetin over various times of (0–24 h). The figure shows a representative result from triplicates and expressed as the mean±S.E. **P<0.01 represents a significant difference between the experimental and control value.

(P<0.01) after 3 h of the treatment, compared to that of untreated cells (4.75 μmol/mg protein). However, quercetin did not affect the malonaldehyde level in BNL CL.2 cells.

3.6. Prooxidant effect of quercetin in normal and transformed cells

The prooxidant effect of quercetin in the hepatic cells was examined by using flow cytometric analysis (Fig. 8).

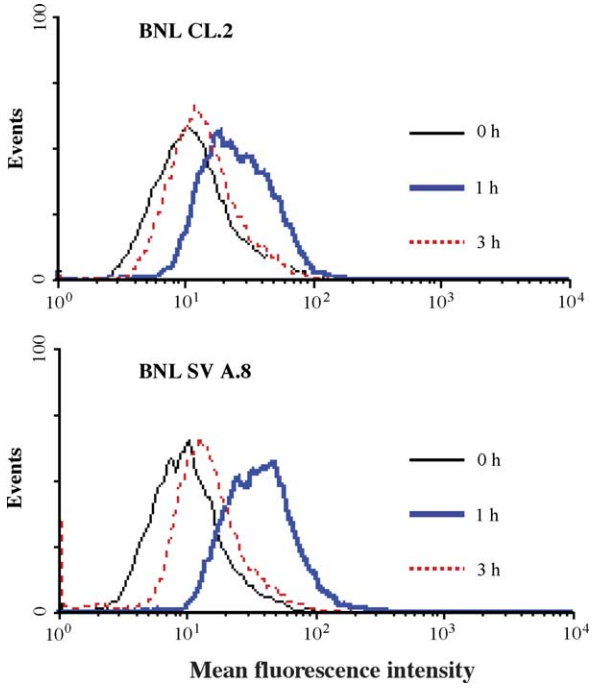

Fig. 8. Flow cytometric analyses of hepatic cells after quercetin treatment. BNL CL.2 (upper) and BNL SV A.8 cells (below) were treated with 100 μM of quercetin for the indicated times. DCFH-DA (25 μM) was then added for an additional 20 min and the fluorescence distribution was analyzed as described in Materials and methods. The figures show a representative result from three different experiments.

When the cells were treated with quercetin, the mean fluorescence intensity, which represents DCF content, increased in both cell types. The largest increase in the DCF level was achieved when the cells were treated with 100 μM of quercetin for 1 h. In particular, the quercetin-mediated increase of DCF content was further sensitive in BNL SV A.8 than BNL CL.2 cells. However, the DCF levels generally decreased during longer periods of treatment, and after 3 h of the treatment, the DCF contents in both cells decreased to a similar level to that of untreated cells.

4. Discussion

In recent years, a plant derived-bioactive substance that is capable of selectively arresting cell growth and inducing apoptosis in tumor cells has received considerable attention in cancer chemopreventive approaches (Paschka et al., 1998; Mukherjee et al., 2001). Enhancement of an antioxidant defense system is also believed to be another mechanism by which many chemopreventive substances alter and reduce cancer risk (Park et al., 2001). In those respects, dietary flavonoid quercetin is thought to be a good material for cancer chemoprevention.

Initially, we evaluated the growth inhibitory effect of quercetin by determining tritium incorporation using normal (BNL CL.2) and transformed hepatic cells (BNL SV A.8). Results from the tritium uptake assay showed that BNL SV A.8 cells were more sensitive to quercetin-mediated inhibition of proliferation than BNL CL. 2 cells (Fig. 1A and B). In addition, trypan blue staining experiment has shown that the viability of BNL SV A.8 cells was much more susceptible to reduction by quercetin treatment, than was the viability of BNL CL.2 cells (Fig. 2A and B). These results indicated a selective influence of quercetin on transformed BNL SV A.8 cells.

To understand the selective cytotoxic effect of quercetin on normal and transformed cells, quercetin-treated hepatic cells were subjected to apoptosis assays (Fig. 3; Tables 1 and 2). As shown by the increased number of positively stained cells in the TUNEL assay (Fig. 3A) and the appearance of characteristic nuclear DNA ladders (Fig. 3B), quercetin treatment sensitively induced apoptosis in BNL SV A.8 cells, but not in BNL CL.2 cells. This differed effect of quercetin on transformed cells was further confirmed by the migration of many cells to sub-G_1 phase in the BNL SV A.8 cells (Tables 1 and 2).

Among the many promising strategies that can be approached for cancer chemoprevention, selective apoptosis induction is regarded as one of the best ways to remove tumor cells. Since almost all artificial agents currently being used in cancer therapy are known to be toxic and produce severe damage to normal cells, naturally occurring agents that are capable of selective and preferential elimination of cancer cells, by inhibiting cell cycle progression and/or causing apoptosis, are further emphasized as promising therapeutic agents (Gupta et al., 2001). With this regard, our findings from the apoptosis analyses suggest that quercetin could be used as a cancer chemopreventive and therapeutic substance. We assume that the profound apoptotic effect of quercetin in BNL SV A.8 cells is related to the activity of signal transduction molecules, since quercetin has been reported to exert inhibitory effects on protein kinases and some transcriptional factors (Caltagirone et al., 2000; Walker et al., 2000; Aligiannis et al., 2001). However, additional experiments should be performed to determine the exact mechanism(s) by which quercetin induces selective apoptosis induction in hepatic cells that have been transformed.

An antioxidant defense system in living organisms consists mainly of a network of non-enzymatic and enzymatic components and is well developed to control the levels of exo- and endogenous ROS. SOD converts superoxide radical into hydrogen peroxide, whereas GPX and CAT convert hydrogen peroxide into water (Izawa et al., 1996). Especially, MnSOD has a pivotal role in reducing mitochondrial oxidative stress and in maintaining GSH content (Hayes and McLellan, 1999; Wheeler et al., 2001). GPX family uses GSH as a cofactor to destroy hydrogen peroxide and lipoperoxides, whereas GR acts to reduce glutathione disulfide (GSSG) to GSH using NADPH as a cofactor (Li et al., 2000). It was reported that quercetin increased the activity of intracellular antioxidants and protected against ethanol-induced oxidative stress in mice liver (Molina et al., 2003). Furthermore, the reduction of GSH content is believed to be involved in a number of down-stream events in apoptosis (Watson et al., 1996; van den Dobbelsteen et al., 1996). Therefore, we assume that the selective induction of apoptosis in the hepatic cells might be associated with an altered activity of an intracellular antioxidant system. In order to determine the effects of quercetin on an intracellular antioxidant system, we carried out several enzyme assays (Figs. 4–6). Results revealed that the activities of SOD, CAT, and GR, and intracellular GSH content significantly increased only in the BNL CL.2 cells after quercetin treatment. Especially in the transformed hepatic cell line, BNL SV A.8, MnSOD activity was not maintained, but significantly diminished by quercetin treatment. We believe that quercetin treatment induced responses mediated by different antioxidant defense system in the two cell lines, and the selective induction of apoptosis in BNL SV A.8 cells is closely associated with the nature of this response. However, further detailed experiments should be performed to confirm this speculation because, in the present study, we could not entirely exclude the possibility that the different response of antioxidant defense system might be involved in the difference of survival of the two cell lines.

Malonaldehyde is the major aldehyde products of lipid peroxidation. Since MDA have demonstrated to be mutagenic and react with DNA to form adducts that lead

to DNA damage, the prevention of lipid peroxidation is an essential process in living organisms (Lawrence, 2000; Yoon et al., 2002). Therefore, a reduction of malonaldehyde level within the cells indicates a decreased risk in the oxidant-mediated lipid peroxidation and cytotoxic damage. Results demonstrated that quercetin treatment significantly increased the malonaldehyde level in a time-dependent manner only in BNL SV A.8 cells (Fig. 7). Based on these results, we hypothesized that the increase of malonaldehyde content in the BNL SV A.8 cells is closely related to the quercetin-mediated increase of cytotoxicity. Finally, we measured the prooxidant activity of quercetin in the hepatic cells (Fig. 8). The results showed that DCF content after quercetin treatment increased in both the cell lines. The quercetin-mediated increase of DCF content was further sensitive in BNL SV A.8 than BNL CL.2 cells, although the DCF levels were similar to that of untreated cells following longer periods of treatment. It indicates that quercetin has conflicting roles, i.e., as an antioxidant and as a prooxidant, depending on the redox state of the cell (Lee et al., 2003). In particular, cells exposed to an increased level of oxidizing species elicited a specific antioxidant response, which was dependent on cell type as well as the type and dose of prooxidant (Allen and Tresini, 2000; Meewes et al., 2001). Initiation of this response is thought to be involved in the activation of redox-sensitive transcription factors such as NF-κB and activator protein-1 (Poulsen et al., 2000; Lee et al., 2003). Collectively, we postulate that quercetin could act as a prooxidant in the hepatic cells, but this stimulates a signal pathway that leads to survival or apoptosis differently, depending on the type of cells and prooxidant activity.

In summary, the present study demonstrates that quercetin has selective growth inhibition and apoptosis-inducing effects in tumorigenic hepatic cells of mice and can be considered to be a natural chemotherapeutic agent. It is also believed that the reduction of MnSOD and an increase of lipid peroxidation might be involved in the selective induction of apoptosis by quercetin in BNL SV A.8 cells. However, further detailed studies are required to determine the exact mechanism(s) involved in the quercetin-mediated selective response on transformed cells. Additional experiments using other types of cells or tissues should also be carried out to compare and confirm the selective effects of quercetin. Furthermore, additional investigations using mouse embryonic primary hepatic cells, which could be a proper normal counterpart of cancer cells, might be helpful to confirm the selective effects of quercetin in normal versus tumorigenic hepatic cells.

Acknowledgements

This study was supported by the Fund of Chonbuk National University Hospital Research Institute of Clinical Medicine to Dr. Y.-M. Jeon. Part of this work was conducted using the facilities of the Research Center of Bioactive Materials at Chonbuk National University.

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