Selective Stimulating Effect of Flavonoids on the Antioxidant Defense System in Normal and Transformed Hepatic Cell Lines

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Abstract – Previously, a flavonoid fraction, which consisted mainly of protocatechuic acid, fustin, fisetin, sulfuretin, and butein, here named RCMF (RVS chloroform-methanol fraction), was prepared from a crude acetone extract of *Rhus verniciflua* Stokes (RVS) which is traditionally used as a food additive and as an herbal medicine. In this study, we evaluated the effects of RCMF on the antioxidant defense system using embryonic normal hepatic cell line (BNL CL.2) and its SV40-mediated transformed cell line (BNL SV A.8). This study demonstrates that RCMF selectively stimulated the antioxidant defense system of normal cells, as BNL CL.2 cells proved to be more sensitive to RCMF-mediated increases of superoxide dismutase, catalase, glutathione, and glutathione reductase than BNL SV A.8 cells. In particular, RCMF caused a significant increase in the malonaldehyde content of BNL SV A.8 cells, which is believed to be closely associated with cytotoxicity of RCMF and RCMF-mediated growth inhibition. Collectively, our findings suggest that the flavonoid fraction, RCMF, selectively stimulates the antioxidant defense system in normal rather than hepatic tumor cells.

Keywords – *Rhus verniciflua* Stokes, Flavonoids, Hepatocytes, Antioxidant defense system

Introduction

Flavonoids are a major group of naturally occurring compounds and are present in most plants. They exert a remarkable spectrum of biological activities, and affect basic cell functions, such as proliferation, differentiation, and apoptosis (Formica and Regelson, 1995; Plaumann *et al.*, 1996). Recently, flavonoids have been recognized for having anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, and antiviral activities (Duarte *et al.*, 1993; Gao *et al.*, 1999; Wong and McLean, 1999), and the beneficial effects of flavonoids are believed to be due to the inhibition of the enzymes involved in signal transduction and to their antioxidant properties. Indeed, many studies have shown that flavonoids inhibit PI3-kinase, protein kinase C, protein tyrosine kinase, and some transcriptional factors, and that such inhibition leads to cell growth arrest and tumor cell death (Gamet-Payrastre *et al.*, 1999; Miranda *et al.*, 1999; Yang *et al.*, 1998).

In addition to their antitumor effects, another striking possibility is that flavonoids might improve the antioxidant defense systems of living organisms. A recent report showed that flavonoids increased the activity of intracellular antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione (GSH), and prevented ethanol-induced oxidative stress in a mouse liver (Molina *et al.*, 2003). It was also reported that flavonoids reduced ethanol-induced hepatic steatosis and lipid peroxidation (Kahraman *et al.*, 2003; Mizui *et al.*, 1987). Therefore, it is believed that flavonoids have a cancer chemo-preventive potential and also improve the endogenous antioxidant defense system. However, little is known regarding the regulatory effects of flavonoids on intracellular antioxidant systems in nonmalignant hepatic cells.

Previously, we prepared a purified flavonoid sample that consisted mainly of protocatechuic acid, fustin, fisetin, sulfuretin, and butein from *Rhus verniciflua* Stokes (RVS) and named it RCMF (Lee, 2004; Lee *et al.*, 2004). In preliminary experiments, it was found that RCMF had antioxidant and antitumor activity in lymphoma cell lines. In the present study, we evaluated the stimulating effects of RCMF on the antioxidant defense system through various enzyme assays using embryonic normal and transformed hepatic cell lines. In addition, the growth inhibitory and cytotoxic effects of RCMF in the cells

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were also investigated.

Materials and Methods

Chemicals and laboratory wares – Unless otherwise specified, all chemicals used in this study were purchased from the Sigma Chemical Co. (St. Louis, MO) and all the laboratory wares were from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ).

Preparation of RCMF – A purified flavonoid sample was prepared from a crude acetone extract of RVS wood, according to the previously described procedure (Jung, 1998). Briefly, 5 g of the dried crude extract was dissolved in methanol and processed by silica gel column chromatography (2.9×45 cm, 230-400 mesh). The sample was then eluted with 200 ml of organic solvent mixture [chloroform:methanol (90:10, v/v)]. The eluted solution was collected and lyophilized to 3.7 g (74% of the initial amount). This final sample is henceforth referred to as RCMF (RVS chloroform-methanol fraction). After the RCMF was further purified by HPLC to identify its chemical composition, five prominent peaks were observed. The compounds contained within peaks 1 to 5 were identified as being protocatechuic acid, fustin, fisetin, sulfuretin, and butein, respectively, by comparing the EI-MS and NMR spectra with previously reported data (Jung, 1998; Lee et al., 2002; Zhang et al., 1998). The RCMF was freshly dissolved in dimethylsulfoxide (DMSO) and final concentration of DMSO did not exceed 0.1% (v/v) throughout the experiments. Concentrations used in experiments are expressed in terms of RCMF dry weight (μg/ml).

Cell culture and treatment – Embryonic normal hepatic cell line, BNL CL.2, and SV40-transformed tumorigenic BNL CL.2 cell line, BNL SV A.8, were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT). We used these two cell lines for comparing the effects of certain agents on normal and transformed cells, because these cells originated from the same mouse strain and developmental stage (Patek et al., 1978). Prior to the RCMF treatment, the cultures were switched to a fresh batch of the same medium. At various times after RCMF treatment, the cells were processed to analyze intracellular antioxidant system, lipid peroxidation, and cell proliferation.

Determination of the activity of intracellular antioxidant systems – After being treated with RCMF for various times, the hepatic cells were rinsed in ice-cold PBS and disrupted by repeated freezing and thawing in a buffer solution containing 50 mM of a phosphate buffer

(pH 7.8), 0.1% Triton X-100, and 0.1 mM EDTA. After centrifugation at 15,000×g for 20 min at 4°C, the supernatants were used as hepatic extract. The protein concentration of the extract was determined by the method reported by Bradford (Bradford, 1976).

Total SOD and Mn SOD were determined according to the method described by Misra and Fridovich (1972). CAT was determined using a slight modification of the method developed by Aebi (1984). Briefly, 10 μl of the hepatic extract was mixed with 240 μl of a phosphate buffer in a cuvette prior to adding 250 μl of 66 mM H_2O_2 diluted in a phosphate buffer. Reductions in optical density, indicative of the decomposition of H_2O_2, were measured at 240 nm for 1 min. GPX was determined using a modified version of the method reported by Flohe and Gunzler (1984). To determine GR activity, 50 μl of 2 mM NADPH in 10 mM Tris buffer (pH 7.0) was added to a cuvette containing 50 μl of 20 mM oxidized GSH (GSSG) in a 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 absorbance was measured at 340 nm for 3 min. GR activity was determined by measuring the decrease in the NADPH concentration, and is expressed as nmol of NADPH/min/mg protein. Cellular GSH content was determined using the method described by Dringen and Hamprecht (1996).

Lipid peroxidation assay – This assay was used to determine intracellular malonaldehyde (MDA) level, as described by Ohkawa et al. (1979). Briefly, 100 μl of the hepatic extract was mixed with 50 μl of 8% SDS and incubated for 10 min at room temperature. The mixture was then resuspended in a buffer containing 375 μl of a 20% acetic acid solution (pH 3.5) and 375 μl of 0.6% thiobarbituric acid (TBA), and incubated at 80°C for 60 min. Subsequently, 250 μl of distilled water and 1.25 ml of a butanol:pyridine mixture (15:1, v/v) were added to the mixtures and then centrifuged at 1,000×g for 5 min. MDA levels in supernatant were measured at 532 nm and are expressed as μmol/mg protein using 1,1,3,3-tetraethoxypropane as a standard.

Determination of cytotoxicity – Cellular cytotoxicity induced by RCMF treatment was determined by trypan blue exclusion assay. Briefly, the hepatic cells were cultured in DMEM supplemented with 10% FBS in the presence of 100 μg/ml RCMF for 12 h and 24 h. After incubation, the cells were stained with 0.4% trypan blue and about 100 cells were counted per treatment. The cytotoxicity was calculated as follows: % cytotoxicity = [(total cells - viable cells) / total cells]×100.

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

Statistical analyses – All the data are expressed as a mean±standard error (SE). One-way ANOVA using SPSS (Ver. 10.0) software was used for multiple comparisons, and $P < 0.05$ was considered significant.

Results

Enzyme assays for SOD, CAT, GSH, GPX, and GR were performed to determine whether RCMF altered the intracellular antioxidant defense system in hepatic cells (Table 1). Initially, RCMF treatment significantly increased the level of total SOD ($P < 0.05$) and Mn SOD in BNL CL.2 cells ($P < 0.001$). However, the SOD activities in BNL SV A.8 cells were decreased significantly after RCMF treatment ($P < 0.01$). A significant increase in CAT activity was observed in both the cells types. After 24 h of the RCMF treatment, the CAT activity was augmented 6.08-fold ($P < 0.001$) and 2.98-fold ($P < 0.001$) in BNL CL.2 and BNL SV A.8 cells, respectively, when compared to the control values. In addition, a significant increase was observed in the concentration of GSH in
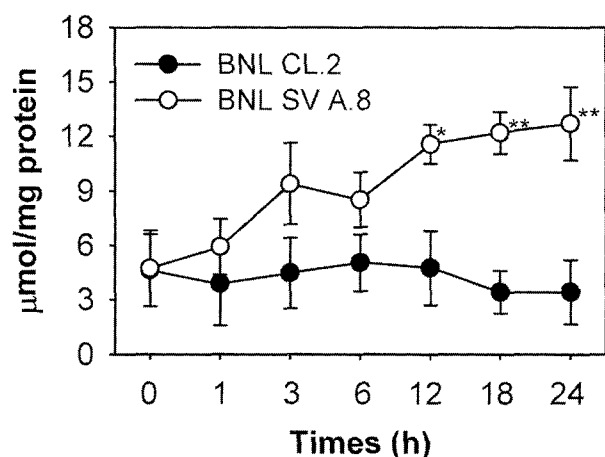
BNL CL.2 cells ($P < 0.001$). However, GPX activity was unaffected by RCMF treatment in either cell type. Finally, RCMF treatment to the hepatic cells caused a significant increase in GR activity only in the BNL CL.2 cells ($P < 0.01$).

A prominent increase in MDA level was observed only in SNL SV A.8 cells in a time-dependent manner (Fig. 1). When the BNL SV A.8 cells were treated with 100 μg/ml RCMF, the intracellular MDA content increased 2.69-fold

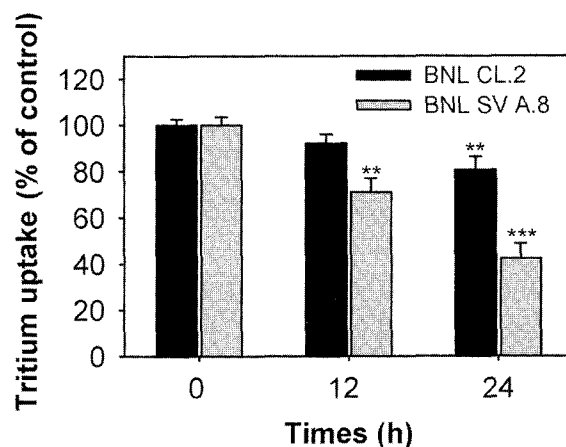**Fig. 2.** Effects of RCMF on DNA synthesis in hepatic cells. BNL CL.2 and BNL SV A.8 cells were treated with 100 μg/ml RCMF for 12 h or 24 h and then incubated with [*methyl*-^{3}H] TdR for the last 12 h of the incubation periods. Representative results from three separate experiments performed in triplicate are shown and bars represent a mean±SE. **$P < 0.01$ and ***$P < 0.001$ represent significant differences between the experimental and control value.

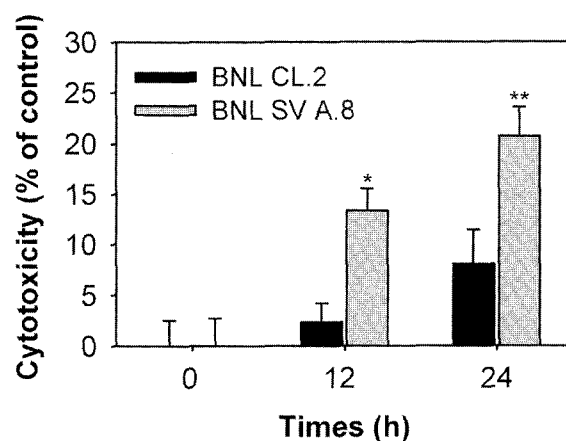**Fig. 1.** Malondialdehyde (MDA) level in hepatic cells treated with RCMF. BNL CL.2 and BNL SV A.8 cells were treated with 100 μg/ml RCMF for various times (0-24 h). The figure shows a representative result from triplicate experiments, and is expressed as the mean±SE. *$P < 0.05$ and **$P < 0.01$ represent significant differences between the experimental and control value.

Fig. 3. Cytotoxic effects of RCMF in hepatic cells. Cells were treated with 100 μg/ml RCMF for the indicated times and then processed for trypan blue staining. Each bar represents a mean±SE of three separate experiments. *$P < 0.05$ and **$P < 0.01$ represent significant differences between the experimental and control value.

$(P < 0.01)$ after 24 h of the treatment, compared to that of the untreated cells (4.72 μmol/mg protein).

The effect of RCMF on cell proliferation was determined by the level of tritium incorporation in the hepatic cells (Fig. 2). The result showed that the BNL SV A.8 cells were more sensitive to the RCMF-mediated inhibition of proliferation. In addition, the RCMF added did not have a substantial cytotoxic effect in the BNL CL.2 cells and the cytotoxicity induced by a 24-h incubation of the cells with 100 μg/ml RCMF was only 8.0% (Fig. 3). However, 20.7% of the BNL SV A.8 cells stained positively with trypan blue when treated with 100 μg/ml RCMF for 24 h.

Discussion

The antioxidant defense system in living organisms is consisted mainly of a network of non-enzymatic and enzymatic components, and is well able to control the exo- and endogenous ROS level. SOD, a component of this system, converts the superoxide radical into hydrogen peroxide, whereas GPX and CAT convert hydrogen peroxide into water (Izawa et al., 1996). In particular, MnSOD has a pivotal role in reducing the mitochondrial oxidative stress and in maintaining the intracellular content of GSH, which is a cofactor for the enzymatic reduction of peroxides and plays an important role in detoxification pathways (Hayes and McLellan, 1999; Wheeler et al., 2001). Moreover, the 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). Therefore, the enhancement of the antioxidant defense system is another mechanism by which bioactive substances may have a chemopreventive effect (Molina et al., 2003; Poulsen et al., 2000).

In the present study, several enzyme assays were performed to investigate the effects of RCMF on the antioxidant defense system. The results obtained showed that RCMF potentially modulates the activities of intracellular antioxidants (Table 1). Initially, RCMF was potent in inducing the activity of MnSOD in BNL CL.2 cells; however, when RCMF was added to BNL SV A.8 cells, MnSOD activity was significantly reduced. On the other hand, CAT activity was significantly enhanced in both cell lines by RCMF treatment. Furthermore, RCMF was highly effective at upregulating intracellular GSH and GR activity, but only in BNL CL.2 cells. These results suggest that the RCMF induces a different response of the antioxidant defense system between the two cell lines, and that RCMF-mediated stimulation of the defense system occurs in BNL CL.2 cells and not in BNL SV A.8 cells.

MDA are major aldehydic products of lipid peroxidation. Since MDA have been 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). Accordingly, a reduction in MDA level in cells indicates a reduced risk of oxidant-mediated lipid peroxidation and cytotoxic damage. Importantly however, the RC

Table 1. Antioxidant enzyme activities in hepatic cells treated with RCMF[a]

Enzymes	Cells[e]	Times (hours)				
		0	3	6	12	24
Total SOD[b]	N	7.24±0.32	7.32±0.33	8.65±0.75	9.08±0.36	12.13±1.23*
	T	10.33±0.74	11.49±0.36	10.34±0.89	11.83±0.58	11.51±0.94
Mn SOD[b]	N	1.83±0.31	2.35±0.44	4.03±0.38***	4.12±0.22***	4.21±0.29***
	T	4.51±0.32	3.86±0.42	3.16±0.31*	2.59±0.24**	2.54±0.24**
CAT[c]	N	2.43±0.19	2.44±0.30	2.66±0.50	8.91±0.82***	14.77±0.60***
	T	3.11±0.43	4.27±0.41	5.00±0.41	6.61±0.61**	9.27±1.55***
GSH[d]	N	2.43±1.09	4.12±0.75	4.29±0.63	4.63±0.29	4.93±0.45*
		1.67±0.63	2.98±0.73	3.04±0.37	3.15±0.60	2.43±0.79
GPX[c]	N	59.92±5.12	81.25±3.98*	75.46±3.98	71.00±3.70	72.00±5.88
	T	57.11±3.42	56.30±2.13	60.73±6.26	52.00±4.55	48.00±10.28
GR[c]	N	26.54±3.41	37.00±6.82	40.22±9.10	54.60±11.37*	65.00±9.10**

[a]Values are mean±SE. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ represent significant differences between the experimental and control value.
[b]Units/mg protein
[c]nmol/min/ml
[d]mmol/mg protein
[e]N and T indicate BNL CL.2 and BNL SV A.8 cells, respectively.

MF treatment to BNL SV A.8 cells caused a significant increase in the MDA level in a time-dependent manner, indicating the induction of lipid peroxidation (Fig. 1). Based on these results, we hypothesized that the significant increase in the MDA content in the BNL SV A.8 cells is closely related to the RCMF-mediated cytotoxicity in the cells.

To evaluate the hypothesis, we examined the growth inhibitory response to RCMF by determining the level of tritium incorporated by BNL CL.2 or BNL SV A.8 cells (Fig. 2). Tritium uptake assay revealed that RCMF treatment actively inhibited tritium incorporation by BNL SV A.8 cells in a dose- and time-dependent manner. However, trypan blue staining showed that the viability of BNL SV A.8 cells treated with 100 µg/ml RCMF for 24 h was approximately 79% of untreated cells (Fig. 3). Therefore, we believe that RCMF caused a cell cycle arrest at either the G_0/G_1 or G_2/M phase, rather than causing cell damage directly, since the trypan blue stains cells with disrupted membranes, whereas viable and apoptotic cells, which have intact membranes, exclude this dye. However, BNL CL.2 cells treated for the same times and with the same RCMF doses showed no significant reduction in cell viability. These results indicated that the BNL SV A.8 cells, but not the BNL CL.2 cells, are sensitive to RCMF-mediated inhibition of cell proliferation. Collectively, our findings suggest that the selective responses to the growth inhibition and MDA induction by the RCMF treatment in the hepatic cells are closely related to the different responses of the antioxidant defense system between the normal and transformed hepatic cells.

In summary, the antioxidant defense system enhancement provides a mechanism by which chemopreventive agents can be used to reduce cancer risk (Park *et al.*, 2001). This study demonstrates that RCMF selectively triggers the stimulation of the antioxidant defense system in normal hepatic cells. However, further detailed *in vivo* and in vitro studies are needed to determine the details of the mechanism involved in the RCMF-mediated selective stimulation of the antioxidant defense system in hepatocytes.

Acknowledgements

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