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Involvement of p53-Mediated Mitochondrial Stress in the Apoptosis Induced by Flavonoids Purified from *Rhus verniciflua* Stokes in Human Osteosarcoma Cells

Song-Woo Chung[1], Seung-Ah Lee[2,3], Jong-Sun Park[1], Kwon-Woo Ryu[1], Mun-Ju Jang[1], Song-Soo Park[1], Choon-Bong Lee[1], Jong-Ghee Kim[1], Young-Mi Jeon[1], and Jeong-Chae Lee[1,3,]*

[1]*Laboratory of Cell Biology in Department of Orthodontics and Institute of Oral Bioscience, Chonbuk National University, Chonju 561-756, Korea*
[2]*Department of Nursing, Chonnam Techno College, Chonnam 516-911, Korea*
[3]*Department of Bioactive Materials, Research Center of Bioactive Materials, Chonbuk National University, Chonju 561-756, Korea*

Abstract – Dietary flavonoids have antioxidant and antitumor promoting effects. *Rhus verniciflua* Stokes (RVS) is a flavonoid-rich herbal medicine and has long been used as a food additive and an antitumor agent in Korea. Previous study demonstrated that a purified flavonoid fraction prepared from RVS, herein named RCMF (the RVS chloroform-methanol fraction), exhibited growth inhibition and induced apoptosis in human osteosarcoma (HOS) cells. This study evaluated if p53-mediated pathway is associated with the RCMF-induced apoptosis in HOS cells. RCMF was shown to be capable of inducing apoptosis of the cells, as expected, and transparently increased p53 expression in the cells. However, the RCMF-induced cytotoxicity was suppressed by transfecting the cells with antisense p53 oligonucleotide, which also inhibited the decrease of Bcl-2 and the increase of Bax in mitochondria, and the release of cytochrome *c* into cytosol. This finding suggests that p53-mediated mitochondrial stress is required for RCMF-induced apoptosis in HOS cells.
Keywords – flavonoids, human osteosarcoma cells, apoptosis, p53, mitochondrial stress

Introduction

Flavonoids are bioactive substances commonly found in most plants and are also integral parts of the human diet (Gamet-Payrastre *et al.*, 1999). The beneficial effects of flavonoids have been attributed to the inhibition of the enzymes involved in signal transduction and to their antioxidant properties (Yang *et al.*, 1998; Lee *et al.*, 2003). Previously, a purified flavonoid fraction, consisting mainly of protocatechuic acid, fustin, fisetin, sulfuretin, and butein, herein named RCMF (RVS chloroform-methanol fraction), was prepared from *Rhus verniciflua* Stokes (RVS) which is traditionally used as a food additive and an herbal medicine (Son *et al.*, 2005).

Our previous studies revealed that RCMF had antitumor activity in lymphoma and hepatoma cell lines (Lee *et al.*, 2004; Son *et al.*, 2005). In addition, RCMF treatment induced growth inhibition and apoptosis in human osteosarcoma (HOS) cells (Jang *et al.*, 2005). The activation of caspase-8 and Bax, the inhibition of Bcl-2 induction, and the cleavage of poly (ADP ribose) polymerase (PARP) were appeared to be associated with the RCMF-mediated apoptosis in the cells. However, the precise mechanisms involved in RCMF-mediated apoptosis of HOS cells are still unclear. Therefore, this study was conducted to further examine the mechanisms and suggested that p53-mediated mitochondrial stress was closely related to the RCMF-mediated apoptosis in HOS cells.

*Author for correspondence
Tel : +82-63-270-4049; E-mail: leejc88@chonbuk.ac.kr

Experimental

Chemicals and laboratory wares – Unless otherwise specified, all chemicals and laboratory wares were obtained from the Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively. A purified flavonoid sample, RCMF, was prepared from a crude acetone extract of RVS wood, according to previously described procedures (Son *et al.*, 2005). The RCMF was freshly dissolved in absolute ethanol prior to use, and the final concentration of ethanol was not in excess of 0.1% (v/v) at any time during the experiments.

Cell culture and treatment – The human osteosarcoma cell line HOS (ATCC, CRL1543) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS HyClone, Logan, UT, USA) and antibiotics. One million cells per milliliter were resuspended in either 2 ml or 100 μl media for spreading onto either 6-well or 96-well flat-bottomed plates, respectively. When cells had reached 70-80% confluence, cultures were replaced to a fresh batch of the same medium with and without 50 μg/ml RCMF. At various times after the treatment, cells were processed for the analyses of cytotoxicity and apoptosis.

Measurement of MTT-reducing activity – At various times after treating the cells with 50 μg/ml RCMF, 5 μl of MTT solution (5 mg/ml in PBS as stock solution) was added into each well, and the cells were further incubated for 4 h at 37 °C. Thereafter, 70 μl of acidic isopropanol was added to each well, and the plates were read at 560 nm using a SpectraCount™ ELISA reader (Packard Instrument Co., Downers Grove, IL, USA).

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay – After exposure to RCMF for various times, HOS cells were fixed with 1% buffered formaldehyde (pH 7.5) on ice for 30 min and then incubated at 37 °C for 30 min in a TdT buffer containing 30 mM Tris-HCl (pH 7.2), 140 mM sodium cacodylate, 1 mM $CoCl_2$, 0.05 mg/ml BSA, 0.1 mM DTT, 7.5 U/ml TdT, and 0.4 nM/ml FITC-5-dUTP. Finally, the cells were washed with PBS and observed under fluorescence microscopy (Axioskop 2, Carl Zeiss, Germany).

Propidium iodide (PI) staining – The suspension (2×10^6 cells) of RCMF-treated HOS cells was fixed with 80% ethanol at 4 °C for 24 h, and then incubated overnight at 4 °C with 1 ml of the PI 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 PI in 1.12% sodium citrate). After staining, 1×10^4 cells were flow cytometrically analyzed using the FACS Calibur system (Becton Dickinson, San Jose, CA, USA).

Western blot analyses – Cell lysates were made in a NP-40 lysis buffer (30 mM Tris-Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 1% NP-40) (Maulik *et al.*, 1998), and protein content was quantified according to the Bradford method (Bradford, 1976). Equal amounts of the protein (40 μg/sample) were separated by 15% SDS-PAGE and blotted onto PVDF membranes. The membranes were blocked for 1 h with 50 mM Tris (pH 7.5) containing 500 mM NaCl, 1% BSA, and 5% non-fat dried milk, and the blots were probed with primary antibodies for 2 h at room temperature or overnight at 4 °C. The membrane was washed three times with a blocking buffer and incubated with second antibodies for 1 h. After washing, the blots were developed with enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and exposed to X-ray film (Eastman-Kodak, Rochester, NY, USA). The polyclonal antibodies specific for p21 (SC-397) and Bcl-2 (SC-783), and the monoclonal antibodies specific for cytochrome *c* (SC-13156) and Bax (SC-7480) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal antibodies specific to p53 (OP03) and α-tubulin were purchased from Oncogene (Boston, USA) and Sigma Chemical Co., respectively.

Transfection with nonsense (NSO) or antisense p53 oligonucleotide (ASO) – HOS cells were seeded at 5×10^5 cells/well in 6 well plates and transfected with NSO (5'-GGAGCCAGGGGGGAGCAGGG-3') or ASO (5'-CCCTGCTCCCCCCTGGC TCC-3'). Each transfection was performed using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) according to the manufacturer's protocol. After 24 h of transfection, cells were exposed to 50 μg/ml RCMF for various times and then processed for MTT and Western blot analyses.

Statistical analyses – All the data are expressed as mean standard error (SE). A one-way ANOVA using SPSS ver. 10.0 software was used for multiple comparisons. A difference of $P < 0.05$ was considered significant.

Results

RCMF-treated HOS cells were subjected to apoptosis assays, including TUNEL assay and PI staining of nuclei (Fig. 1). A time-dependent increase in the number of positively stained HOS cells after the RCMF treatment was found by TUNEL staining (upper panel). When the cells were treated with 50 μg/ml RCMF for 24 h, the level of apoptosis was measured to be 23.2%, while 52.4% of the cells were apoptotic after being treated with 50 μg/ml RCMF for 48 h. RCMF-induced apoptosis was also observed by PI staining. In the absence of RCMF, the proportion of apoptotic HOS cells was only 5.6% of the total cell population while the proportion of apoptotic cells increased in the time-dependent manner when the cells were treated with 50 μg/ml RCMF (lower panel).

In order to determine if there is a relationship between apoptosis induction and cell cycle arrest, the effects of RCMF on the induction of p53 and p21 proteins were evaluated (Fig. 2). As shown in the figure, RCMF stimulated p53 expression in HOS cells, which was dependant

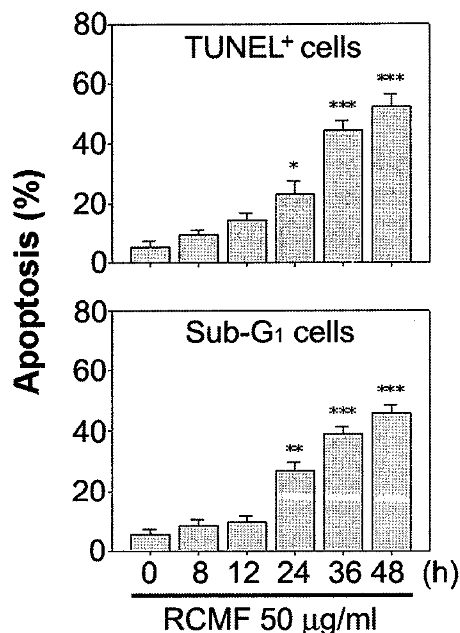

Fig. 1. RCMF induces apoptotic death of HOS cells.
HOS cells were treated with 50 μg/ml RCMF for the indicated times. After incubation, the cells were processed for staining with FITC-conjugated dUTP (upper panel) or for flow cytometric analyses after PI staining (lower panel). Each bar shows the mean ± SE of three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. untreated control cells (ANOVA, Scheffe's test).

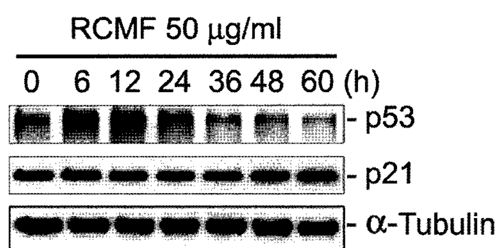

Fig. 2. RCMF leads to p53 induction in HOS cells.
HOS cells were exposed to 50 μg/ml RCMF for various times, and the induction of p53 and p21 was determined by Western blotting as described in the Materials and Methods. A representative data from triplicate experiments is shown.

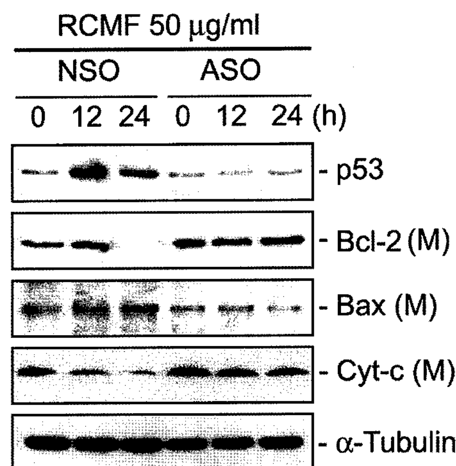

Fig. 3. Western blot analysis of mitochondrial apoptogenic factors after transfection with nonsense or antisense p53 oligonucleotide.
HOS cells were transfected with nonsense and antisense p53 oligonucleotides as described in the Materials and Methods. After 24 h incubation, the cells were treated with 50 μg/ml RCMF for the indicated times, and p53 expression and mitochondrial levels of Bcl-2, Bax, and cytochrome *c* were determined by Western blot analysis. A representative data from triplicate experiments is shown. NSO, nonsense oligonucleotide; ASO, antisense oligonucleotide.

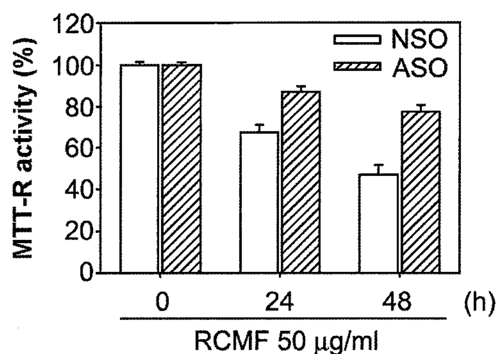

Fig. 4. Induction of p53 is required for the execution of RCMF-mediated cytotoxicity in HOS cells.
HOS cells were transfected with nonsense and antisense p53 oligonucleotides. After 24 h of the transfection, they were treated with 50 μg/ml RCMF for 24 and 48 h, and then processed for MTT assay for determining MTT-reducing activity. Each bar shows the mean ± SE of three separate experiments.

on the times exposed to RCMF. When the cells were treated with 50 μg/ml RCMF, the expression of p53 was dramatically increased 6 h, peaked 12 h, and gradually decreased to basal level 36 h after the treatment. In contrast, the level of p21 expression was not affected by the RCMF treatment in any experimental times studied. In addition, cell cycle analysis revealed that treating the cells with RCMF increased the apoptotic cell populations in a dose-dependent manner without an apparent arrest in cell cycle progression (data not shown). Therefore, the RCMF-mediated apoptosis in HOS cells was thought to be closely related to p53 induction rather than a cell cycle arrest.

To determine the requirement of p53 for RCMF-mediated apoptosis in HOS cells, the effects of transfection with the antisense p53 oligonucleotide were examined. As shown in Fig. 3, this block inhibited the RCMF-mediated changes of mitochondrial apoptogenic factors such that the decrease of Bcl-2, the increase of Bax, and the release of cytochrome *c* into cytosol were prevented. In addition, the transfection inhibited the

RCMF-mediated decrease of MTT-reducing activity (Fig. 4). The results suggest that RCMF transactivates and induces p53 expression that plays a critical role in RCMF-mediated apoptosis of HOS cells.

Discussion

Current studies involved in developing effective cancer-preventive approaches have focused mainly on the utilization of natural bioactive agents that can inhibit proliferation and induce apoptosis in cancer cells (Farr, 1997; Pezutto, 1997; Mukherjee *et al.*, 2001). RCMF is a purified flavonoid fraction and is found to be active substances responsible for the antioxidant and anticancer activity of RVS (Jang *et al.*, 2005; Son *et al.*, 2005).

Flavonoids have been reported to have inhibitory effects on several intracellular kinases and some transcriptional factors, and such inhibition in turn have been shown to arrest cell growth and induce cell death in several carcinoma cell lines (Yang *et al.*, 1998; Gamet-Payrastre *et al.*, 1999; Miranda *et al.*, 1999; Caltagirone *et al.*, 2000). Therefore, it can be postulated that the inhibition of activity of signal transduction molecules involved in the cell cycle is related to the RCMF-induced apoptosis. However, RCMF treatment resulted in a time-dependent accumulation of HOS cells in sub-G_1 phase with a concomitant loss from G_0/G_1 and S phases. No change of p21 expression was also observed, although there was a dramatic increase of p53 expression level. This suggests that RCMF-mediated apoptosis in HOS cells was not related to an arrest of cell cycle progression. In addition, the induction of p53 is thought to play a key role in the RCMF-mediated apoptotic process because the cellular machinery leading to apoptosis can be induced if p53 is overexpressed (Lowe *et al.*, 1993; Muller *et al.*, 1998).

Mitochondria play a critical role in numerous apoptotic responses through the release of mitochondrial apoptogenic proteins such as AIF, EndoG, and cytochrome *c* (Li *et al.*, 2001; Bernhard *et al.*, 2003; Zanna *et al.*, 2005). Members of Bcl-2 family control the release of these proteins. Bcl-2 is an integral membrane protein that prevents apoptosis through the inhibition of the efflux of the proteins whereas Bax and Bid are translocated from the cytoplasm to the outer mitochondrial membrane, where they oligomerize to form pores and trigger apoptosis (Gross *et al.*, 1999; Harris and Thompson, 2000). This study showed that the RCMF-mediated induction of mitochondrial apoptogenic factors and the loss of MTT-reducing activity in HOS cells were inhibited by transfecting the cells with antisense p53 oligonucleotides. Therefore, it is suggested that mitochondrial stress according to the p53-mediated changes of Bcl-2 family is closely related to the apoptosis induced by RCMF in HOS cells.

In summary, many studies have demonstrated that p53 plays an important role in the induction of apoptosis by various anticancer agents (Miyashita and Reed, 1995; Harris, 1996). This study postulated that induction of p53 is a direct mediator in RCMF-mediated apoptosis of HOS cells, although transfection of antisense p53 oligonucleotide did not completely attenuate cell death. Collectively, we suggest that changes of Bcl-2 family in mitochondrial levels is necessary for induction of apoptosis by RCMF, which being closely regulated by RCMF-mediated induction of p53 in HOS cells.

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