Natural Product Sciences
11(1) : 45-49 (2005)

Accelerating Effect of TNF-α on the *Rhus verniciflua*-induced Growth Inhibition and Apoptosis in Human Osteosarcoma Cells

Hyun-Duck Kim[1], Sung-Ho Kook[1], Beom-Tae Kim[2], Jong-Ghee Kim[1], Young-Mi Jeon[1], and Jeong-Chae Lee[1,2,*]

[1]Laboratory of Cell Biology in Department of Orthodontics and Institute of Oral Bioscience,
[2]Research Center of Bioactive Materials, Chonbuk National University, Chonju 561-756, Korea

Abstract – Previously, a flavonoid fraction, which consisted mainly of protocatechuic acid, fustin, fisetin, sulfuretin, and butein, here named RCMF [*Rhus verniciflua* Stokes (RVS) chloroform-methanol fraction], was prepared from a crude acetone extract of RVS which is traditionally used as a food additive and as an herbal medicine. In the present study, we investigated the effects of TNF-α on RCMF-induced growth inhibition and apoptosis induction using human osteosarcoma (HOS) cells. The results from tritium uptake and MTT assays showed that TNF-α treatment itself (10 ng/ml) did not induce any cytotoxicity, but it actively accelerated RCMF-mediated cytotoxicity of HOS cells. RCMF-induced cytotoxicity and its facilitation by TNF-α was verified to be apoptotic, based on the increased DNA fragmentation and low fluorescence intensity in nuclei after propidium iodide (PI) staining of HOS cells. This speculation was further demonstrated by monitoring the Annexin V/PI double staining which could discriminate the difference between apoptotic and necrotic deaths. Collectively, our findings indicate that TNF-α accelerates RCMF-induced cytotoxicity in HOS cells.
Keywords – *Rhus verniciflua* Stokes, flavonoids, human osteosarcoma cells, growth inhibition, apoptosis

Introduction

Bone cells and their interactions are very sensitive to systemic and local factors such as hormones and cytokines. Among the factors, tumor necrosis factor-α (TNF-α) has been known to play a pivotal role in bone metabolic diseases. TNF-α stimulates osteoblasts to secrete other inflammatory cytokines such as interleukin (IL)-1 and IL-6, and prostaglandin E_2 (PGE_2) as well as TNF-α itself, which act directly on osteoclasts to cause bone resorption (Franchimont *et al.*, 1997; Glantschnig *et al.*, 2003). In addition, TNF-α induces apoptosis of osteoblasts (Chua *et al.*, 2002; Suh *et al.*, 2003). Therefore, an increase of TNF-α level and a decrease in osteoblast cell numbers via apoptosis could be responsible for bone loss, and if these conditions persist and prolong, osteoporosis could occur.

On the other hand, flavonoids are commonly found in most plants and are integral parts of human diet (Gamet-Payrastre *et al.*, 1999). Flavonoids affect various cell functions, such as growth (Formica and Regelson, 1995), differentiation (Plaumann *et al.*, 1996), and apoptosis (Caltagirone *et al.*, 2000). Flavonoids have been recognized for having numerous pharmacologic activities including anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, and antiviral properties (Duarte *et al.*, 1993; Gao *et al.*, 1999; Wong and McLean, 1999). In recent years, there has been a global trend toward the use of natural bioactive compounds as chemoregulators of bone cells (Choi and Koo, 2003; Suh *et al.*, 2003; Gallagher *et al.*, 2004). Considering the chemical properties of flavonoids, we postulated that dietary antioxidant flavonoids could inhibit TNF-α-mediated bone diseases and regulate the balanced coupling of osteoblasts and osteoclasts. However, effects of TNF-α on osteosarcoma cells have not been widely reported. In addition, effects of flavonoid on TNF-α-untreated or -treated osteosarcoma cells are not investigated.

Previously, a flavonoid fraction, which consisted mainly of protocatechuic acid, fustin, fisetin, sulfuretin, and butein, herein named RCMF [*Rhus verniciflua* Stokes (RVS) chloroform-methanol fraction], was prepared from a crude acetone extract of RVS, which is traditionally used as a food additive and as an herbal medicine (Son *et al.*, 2005). In this study, we investigated the effects of RCMF on TNF-α-untreated or -treated osteosarcoma cells.

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

Experimental

Chemicals and laboratory wares – Unless otherwise specified, all chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO) and all laboratory wares were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ). A purified flavonoid sample, RCMF, was prepared from a crude acetone extract of RVS wood, according to previously described procedures (Son *et al.*, 2005). RCMF was freshly dissolved in absolute ethanol and final concentration of ethanol did not exceed 0.1% (v/v) at any time during experiments. Concentrations used in experiments are expressed in terms of RCMF dry weight (μg/ml).

Cell culture and treatment – 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) and antibiotics. One million cells per milliliter were resuspended in either 2 ml or 100 μl media for spreading onto either 35-mm culture dishes or 96-well flat-bottomed plates, respectively. Before RCMF and/or TNF-α treatment, cultures were switched to a fresh batch of the same medium. At various times after treatment, cells were processed for the analyses of cell growth, cytotoxicity, and apoptosis.

Measurement of DNA synthesis – The level of DNA synthesis by HOS cells after RCMF and/or TNF-α 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 16 h of culture periods. Cells were then collected with a cell harvester (Inotech Inc., Switzerland), and the tritium contents were measured using a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL).

Measurement of cell viability – 3-(4,5-Dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide (MTT) was used for evaluation of viability of the cells. HOS cells were cultured in DMEM supplemented with 10% FBS and antibiotics in the presence of 1 to 50 μg/ml RCMF and/or 10 ng/ml TNF-α for various times. Thereafter, 10 μl of MTT solution (5 mg/ml in PBS as stock solution) was added into each well, and then the cells were further incubated for 4 h at 37°C. To measure the absorbance, 70 μl of acidic isopropanol was added to each well and absorbance was read at 560 nm using a SpectraCount™ (Packard Instrument Co.) ELISA reader.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay – After exposure to 10 ng/ml TNF-α in the presence of RCMF (1-50 μg/ml) for various times, HOS cells were fixed with 1% buffered formaldehyde (pH 7.5) on ice for 30 min. Cells were then washed with PBS, resuspended in 70% ice-cold ethanol, and kept at -20°C for 1 h. Cells were then rehydrated with PBS and incubated at 37°C for 30 min in 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, cells were washed with PBS and observed under a fluorescence microscope (Axioskop 2, Carl Zeiss, Germany).

Propidium iodide (PI) staining – RCMF-induced DNA fragmentation was also determined by flow cytometric analysis after PI staining. Initially, suspension (2×10^6 cells) of RCMF-and/or TNF-α-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 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).

Annexin V/PI double staining – RCMF-mediated cytotoxicity of HOS cells was also examined through double staining method with Annexin V and PI (Mesner and Kaufmann, 1997). Briefly, 1 μg/ml of FITC-labeled Annexin V in HEPES buffer (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4) containing 1 μg/ml of PI was added to cells and incubated for 30 min at room temperature. After staining, cells (1×10^4 cells) were flow cytometrically analyzed using the FACS Calibur® system (Becton Dickinson).

Statistical analyses – All 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 and Discussion

TNF-α has been known to play a pivotal role in bone metabolic regulation such that TNF-α stimulates osteoblasts to secrete other inflammatory cytokines and TNF-α itself (Franchimont *et al.*, 1997; Glantschnig *et al.*, 2003), and also induces apoptosis of osteoblasts (Chua *et al.*, 2002; Suh *et al.*, 2003). Otherwise, flavonoids exert various pharmacological properties including anti-inflammatory and antioxidant activities. This let us to postulate that flavonoids have a protective effect on TNF-α-induced cytotoxicity. However, effects of TNF-α on osteosarcoma cells were not widely evaluated. Moreover, effects of

flavonoids in the presence of TNF-α have not been investigated.

Tritium uptake assay showed that TNF-α treatment alone (10 ng/ml) did not reduce DNA synthesis by HOS cells (Fig. 1). In contrast, presence of TNF-α stimulated RCMF-induced inhibition of tritium incorporation in the cells. Similar to tritium uptake assay, TNF-α alone did not reduce the viability of HOS cells, but it clearly accelerated RCMF-induced cytotoxicity of the cells (Fig. 2). Following a 72 h incubation with 50 μg/ml RCMF, cell viability of HOS cells was determined by 57%, whereas in the presence of 10 ng/ml TNF-α, it

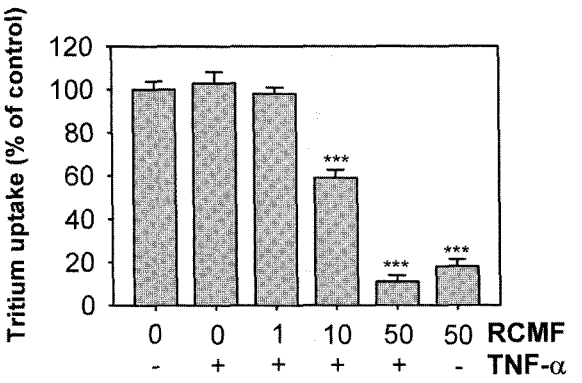

Fig. 1. Effects of TNF-α on RCMF-induced decrease of DNA synthesis in HOS cells. HOS cells were treated with various concentrations (0-50 μg/ml) of RCMF in the absence or presence of 10 ng/ml TNF-α for 72 h and then incubated with [methyl-^{3}H] TdR for the last 16 h of incubation periods. A representative result from three separate experiments is shown and each bar represents the mean±SE of experiments performed in triplicates. $^{***}P < 0.001$ represents significant difference between the experimental and control values.

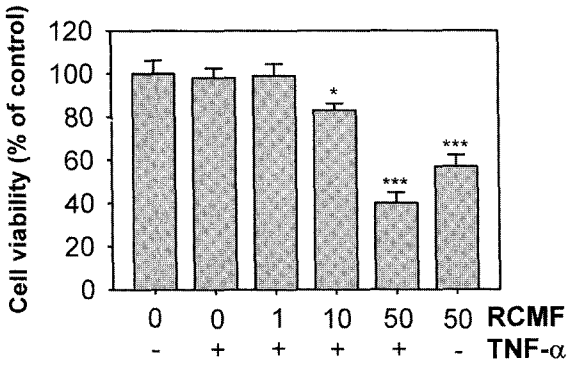

Fig. 2. Effects of TNF-α on RCMF-induced decrease of cell viability in HOS cells. HOS cells were treated with the indicated concentrations (0-50 μg/ml) of RCMF in the presence or absence of 10 ng/ml TNF-α for 72 h and then incubated for another 4 h in the presence of 10 ml of MTT solution (5 mg/ml in PBS as stock solution). A representative result from three separate experiments is shown and each bar represents the mean±SE of experiments performed in triplicates. $^{*}P < 0.05$ and $^{***}P < 0.001$ represent significant differences between the experimental and control values.

decreased to 40% of the level in untreated cells. Considered these results, it was suggested that in the presence of TNF-α, RCMF-induced growth inhibition and cytotoxicity of HOS cells were accelerated.

Apoptosis is the result of highly complex cascade of

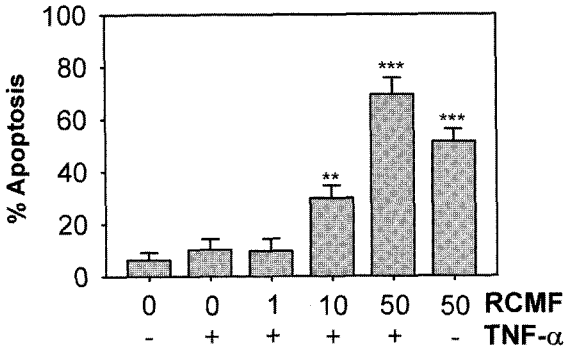

Fig. 3. Effects of TNF-α on RCMF-induced apoptosis of HOS cells. HOS cells were treated with different concentrations (0-50 μg/ml) of RCMF in the presence or absence of 10 ng/ml TNF-α for 72 h. After incubation, cells were stained with FITC-conjugated dUTP and the degree of apoptosis was assessed. Each bar shows the mean±SE of three separate experiments and $^{**}P < 0.01$ and $^{***}P < 0.001$ represent significant differences between the experimental and control values.

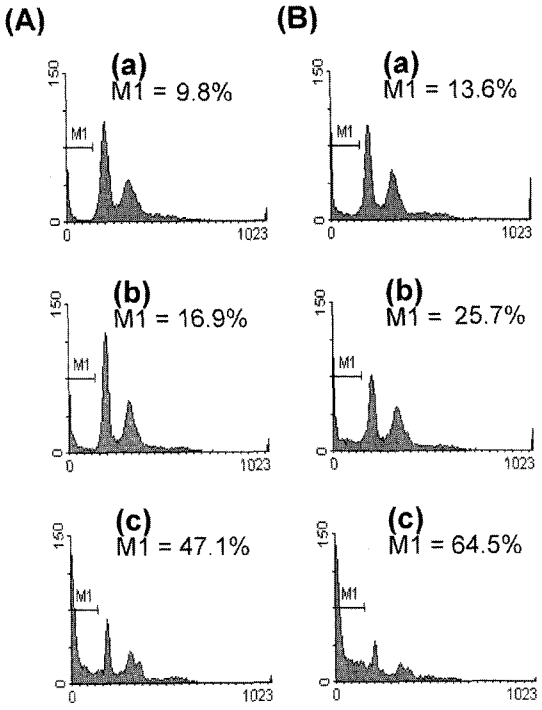

Fig. 4. Flow cytometric analyses of TNF-α-exposed HOS cells in the presence of RCMF. (A) The cells were incubated for 72 h without (a) or with 10 (b) and 50 μg/ml RCMF (c). (B) The cells were treated with 10 ng/ml TNF-α in the absence (a) and presence of 10 (b) and 50 μg/ml RCMF (c). The figures show a representative staining profile for 10,000 cells per experiment. M1 is the cell population defined as apoptotic.

Table 1. Flow cytometric analyses of HOS cells after Annexin V/PI double staining

Populations	0	0	10	50	50	RCMF (μg/ml)
	-	+	+	+	-	TNF-α (10 ng/ml)
Annexin V^-/PI^-	94.1±2.8	87.2±2.9	62.7±3.4	22.4±3.7	40.1±2.4	
Annexin V^+/PI^-	3.9±2.4	9.8±2.8	33.7±3.5	71.8±3.3	53.8±2.7	
Annexin V^-/PI^+	0.2±0.1	1.2±0.1	1.2±0.1	2.5±0.1	2.6±0.3	
Annexin V^+/PI^+	1.8±0.3	1.8±0.2	2.4±0.1	3.3±0.4	3.5±0.6	

HOS cells were incubated with the indicated doses of RCMF for 72 h in the presence or absence of TNF-α. The percentage of cells was calculated through WinMDI 2.8 program of Annexin V and PI fluorescence data. Data represent the mean±SD of experiments performed in triplicates. Annexin V^-/PI^-, viable non-apoptotic cells; Annexin V^+/PI^-, apoptotic cells; Annexin V^-/PI^+, necrotic cells; Annexin V^+/PI^+, necrotic cells.

cellular events that characterized in chromatin condensation, DNA fragmentation, cytoplasmic membrane blebbing, and cell shrinkage (Benson *et al.*, 1996; Allen *et al.*, 1997). To understand the nature of TNF-α-mediated facilitation of RCMF-induced cytotoxicity of HOS cells, cells were subjected to apoptosis assays, including TUNEL assay and PI staining of nuclei (Figs. 3 and 4). We noted a marked increase in the number of RCMF-mediated positively stained cells by TUNEL staining after TNF-α treatment (Fig. 3). When HOS cells were treated for 72 h with 50 μg/ml RCMF alone, apoptotic cell population was measured to be 51.3%, while in the presence of 10 ng/ml TNF-α, it was further augmented and determined as 69.4%. However, TNF-α treatment itself did not show any significant induction of apoptotic cell death. Promoting effect of TNF-α on RCMF-induced apoptosis was supported by flow cytometric analyses after PI staining (Fig. 4). In the absence of TNF-α, apoptotic HOS cells were 47.1% of total cell population after 50 μg/ml RCMF treatment for 72 h (Fig. 4A-c). However, apoptotic cells further increased when RCMF-treated cells were exposed to TNF-α, such that about 64% of HOS cells were apoptotic when the cells were treated with 50 μg/ml RCMF and 10 ng/ml TNF-α for 72 h (Fig. 4B-c).

To further understand whether TNF-α-mediated acceleration of RCMF-induced cytotoxicity in HOS cells are mediated by apoptosis or necrosis, Annexin V/PI double staining was performed (Table 1). Early stage of apoptosis might be implicated in the loss of membrane phospholipid asymmetry and appearance of phosphatidyl serine on the outer surface of cells, which should be found in the inner leaflet of plasma membrane in normal cells and highly stained with Ca^{2+}-dependent phospholipid-binding protein Annexin V. Phosphatidyl serine translocation was believed to be the hallmark of apoptosis and the detection of the phosphatidyl serine externalization might be more sensitive method than the measurement of histone-associated DNA fragmentation to indicate the occurrence of apoptosis (Koopman *et al.*, 1994). In addition, the results from PI staining could be utilized as a method to identify necrotic cell death (Vermes *et al.*, 1995). When HOS cells were treated with 10 ng/ml TNF-α, proportion of apoptotic cells (Annexin V^+/PI^-) was very low. However, apoptotic cell populations were dramatically increased after RCMF treatment, such that when the cells were treated with 50 μg/ml RCMF in the presence of 10 ng/ml TNF-α, about 71.8% of the cells were apoptotic. These results suggested that RCMF induced apoptosis in HOS cells and this apoptotic death was further accelerated in the presence of TNF-α. Consequently, we believe that TNF-α does not affect viability of HOS cells at least in the condition studied, and that RCMF can be used as a booster of chemopreventive agent treatment for osteosarcoma. However, further detailed experiments are needed to elucidate the contribution of RCMF and/or TNF-α to osteosarcoma *in vivo* as well as *in vitro*.

Acknowledgements

This study was supported by a grant, from the Korea Health 21 R&D Project, the Ministry of Health & Welfare, Republic of Korea (03-PJ1-PG3-22000-0001), to Dr. J.-C. Lee. Dr. K.-Y. Lee was supported by the Chonbuk National University Post-Doctoral Fellowship Program.

References

Allen, R.T., Hunter, W.J. 3rd., and Agrawal, D.K., Morphological and biochemical characterization and analysis of apoptosis. *J. Pharmacol. Toxicol. Methods* **37**, 215-228 (1997).

Benson, R.S., Heer, S., Dive, C., and Watson, A.J., Characterization of cell volume loss in CEM-C7A cells during dexamethasone-induced apoptosis. *Am. J. Physiol.* **270**, C1190-1203 (1996).

Caltagirone, S., Rossi, C., Poggi, A., Ranelletti, F.O., Natali, P.G., Brunetti, M., Aiello, F.B., and Piantelli, M., Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. *Int. J. Cancer* **87**, 595-600 (2000).

Choi, E.M., and Koo, S.J., Effects of soybean ethanol extract on

the cell survival and oxidative stress in osteoblastic cells. *Phytother. Res.* **17**, 627-632 (2003).

Chua, C.C., Chua, B.H., Chen, Z., Landy, C., and Hamdy, R.C., TGF-beta1 inhibits multiple caspases induced by TNF-alpha in murine osteoblastic MC3T3-E1 cells. *Biochim. Biophys. Acta* **1593**, 1-8 (2002).

Duarte, J., Perez-Vizcaino, F., Zarzuelo, A., Jimenez, J., and Tamargo, J., Vasodilator effects of quercetin in isolated rat vascular smooth muscle. *Eur. J. Pharmacol.* **239**, 1-7 (1993).

Formica, J.V., and Regelson, W., Review of the biology of quercetin and related bioflavonoids. *Food Chem. Toxicol.* **33**, 1061-1080 (1995).

Franchimont, N., Rydziel, S., Delany, A.M., and Canalis, E., Interleukin-6 and its soluble receptor cause a marked induction of collagenase 3 expression in rat osteoblast cultures. *J. Biol. Chem.* **272**, 12144-12150 (1997).

Gallagher, J.C., Satpathy, R., Rafferty, K., and Haynatzka, V., The effect of soy protein isolate on bone metabolism. *Menopause* **11**, 290-298 (2004).

Gamet-Payrastre, L., Manenti, S., Gratacap, M.P., Tulliez, J., Chap, H., and Payrastre, B., Flavonoids and the inhibition of PKC and PI3-kinase. *Gen. Pharmacol.* **32**, 279-286 (1999).

Gao, Z., Huang, K., Yang, X., and Xu, H., Free radical scavenging and antioxidant activities of flavonoids extracted from the radix of *Scutellaria baicalensis* Georgi. *Biochim. Biophys. Acta* **1472**, 643-650 (1999).

Glantschnig, H., Fisher, J.E., Wesolowski, G., Rodan, G.A., and Reszka, A.A., M-CSF, TNFalpha and RANK ligand promote osteoclast survival by signaling through mTOR/S6 kinase. *Cell Death Differ.* **10**, 1165-1177 (2003).

Koopman, G., Reutelingsperger, C.P., Kuijten, G.A., Keehnen, R.M., Pals, S.T., and van Oers, M.H., Annexin V for flow cytometric detection of phosphatidyl- serine expression on B cells undergoing apoptosis. *Blood* **84**, 1415-1420 (1994)

Plaumann, B., Fritsche, M., Rimpler, H., Brandner, G., and Hess, R.D., Flavonoids activate wild-type p53. *Oncogene* **13**, 1605-1614 (1996).

Son, Y.O., Lee, K.Y., Lee, J.C., Jang, H.S., Kim, J.G., Jeon, Y.M., and Jang,Y.S., Selective antiproliferative and apoptotic effects of flavonoids purified from *Rhus verniciflua* Stokes on normal versus transformed hepatic cell lines. *Toxicol. Lett.* **155**, 115-125 (2005).

Suh, K.S., Koh, G., Park, C.Y., Woo, J.T., Kim, S.W., Kim, J.W., Park, I.K., and Kim, Y.S., Soybean isoflavones inhibit tumor necrosis factor-alpha-induced apoptosis and the production of interleukin-6 and prostaglandin E_2 in osteoblastic cells. *Phytochemistry* **63**, 209-215 (2003).

Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C., A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J. Immunol. Methods* **184**, 39-51 (1995).

Wong, W.S., and McLean, A.E., Effects of phenolic antioxidants and flavonoids on DNA synthesis in rat liver, spleen, and testis *in vitro*. *Toxicology* **139**, 243-253 (1999).

(Accepted March 9, 2005)