Int J Clin Exp Med 2016;9(2):2437-2443
www.ijcem.com /ISSN:1940-5901/IJCEM0015661

Original Article

Menadione induces apoptosis in a gastric cancer cell line mediated by down-regulation of X-linked inhibitor of apoptosis

Min Ho Lee[1], Ji Yeong Yang[1], Yoonjung Cho[1], Min Park[1], Hyun Jun Woo[1], Hyun Woo Kim[1], Hye Jin Kwon[1], Sa-Hyun Kim[2], Cheol Moon[2], Tae Ue Kim[1], Jong-Bae Kim[1]

[1]Department of Biomedical Laboratory Science, College of Health Sciences,Yonsei University, Wonju 26493, Republic of Korea; [2]Department of Clinical Laboratory Science, Semyung University, Jaecheon 390-711, Republic of Korea

Received September 6 2015; Accepted January 7, 2016; Epub February 15, 2016; Published February 29, 2016

Abstract: Recent studies have reported menadione (vitamin K3) has inhibitory effect on several types of cancer cells including lung cancer, breast cancer, ovarian cancer, pancreatic cancer and hepatocellular carcinoma, although the effect of menadione on gastric cancer cells is unclear. Therefore, we have investigated the inhibitory effect and the mechanism of menadione on gastric cancer cells. We found that cell viability of AGS (gastric cancer cell) was dramatically inhibited by menadione, while HS738 (non-cancerous gastrointestinal cell) was not influenced by the same dose of menadione treatment. Annexin V-FITC assay results suggested that decreased cell viability was due to the induction of apoptosis and it was confirmed by the results indicating activation of caspase-3 and caspase-9 and cleavage of PARP in the Westernblot. We further investigated the upstream regulatory molecules involved in the apoptosis and discovered that manadione reduces expression of X-linked inhibitor of apoptosis protein (XIAP). XIAP is a potent regulator of apoptosis which binds to and inhibits activity of caspases there by suppressing apoptosis. These results indicate that menadione activates caspase cascade and induces apoptosis mediated by down-regulation of XIAP expression in gastric cancer cells.

Keywords: Menadione, XIAP, gastric cancer, apoptosis

Introduction

Menadione (vitamin K3) is a synthetic form of vitamin K which possesses strong bioactivity. Previous reports considered menadione as an apoptosis inducing and anti-tumor factor. Menadione has been shown to induce the apoptotic cell death on several types of cancer cells including lung cancer, breast cancer, hepatocellular carcinoma, pancreatic cancer and ovarian cancers [1-6].

Al-Suhaimi *et al.* confirmed that menadione induces cell apoptosis by activation of caspase-3 signal on hepatocellular carcinoma cells [4]. Osada *et al.* evaluated the efficacy of menadione against pancreatic cancer by caspase-3 activation and PARP cleavage [2]. Ovarian cancer cells treated with menadione showed a decrease in Bid, Bcl-2, Bcl-xL, and survivin protein levels; an increase in Bax levels; a release of cytochrome c; activation of caspases (-8, -9, and -3); and cleavage of PARP-1 [5].

Menadione is a well-known anti-tumor factor which has potential role against many cancer cell lines. However, the effect of menadione on gastric cancer cells has not been studied. In human gastric cancer cell lines, we therefore conducted the suppressing effect of menadione as well as evaluation of the molecular signaling involved in apoptosis.

Materials and methods

Materials

RPMI164, DMEM, fetal bovine serum (FBS), streptomycin-penicillin, and trypsin-EDTA were

Table 1. List of primers used in the experiments

Primers	Sequences (5'-3')		Product	Annealing	Cycles
	Forward	Reverse	Length (bp)	Temperature (°C)	
XIAP	CAACACTGGCACGAGCAGGGT	ACATGGCAGGGTTCCTCGGGT	348	55	25
BCL2	CATTTCCACGTCAACAGAATTG	AGCACAGGATTGGATATTCCAT	505	55	35
BAX	AAGAAGCTGAGCGAGTGTC	CGGCCCCAGTTGAAGTTGC	158	60	30
BAK	GCCCAGGACACAGAGGAGGTT	AAACTGGCCCAACAGAACCACACC	527	60	35
GAPDH	CGGGAAGCTTGTCATCAATGG	GGCAGTGATGGCATGGACTG	349	55	20

obtained from Gibco (Carlsbad, CA, USA). Annexin V-FITC apoptosis detection kit I was purchased from BD Biosciences (Sparks, MD, USA). EZ-Cytox cell viability assay kit was purchased from Daeil Lab Service (Seoul, Korea). Trizol reagent, random hexamer, and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) were purchased from Invitrogen (Carlsbad, CA, USA). Protease inhibitor cocktail were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Antibodies to detect caspase-3, caspase-7, caspase-9, PARP, BAX, BAK and XIAP were purchased from Cell Signaling Technology (Danvers, MA, USA) and -actin antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell culture

AGS cells (ATCC CRL-1739, Manassas, VA, USA) were cultured in RPMI1640 medium supplemented with 10% FBS and streptomycin-penicillin. HS738 cells (ATCC CRL-7869) were cultured in DMEM medium supplemented with 10% FBS and streptomycin-penicillin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Cell viability assay

Cells (1×10^4 per well) were plated in 96-well plates. After 24 h, cells were treated with various concentrations of menadione. The cells were then incubated for 24 h and subjected to WST assay by using EZ-Cytox cell viability assay kit according to manufacturer's instruction. Briefly, 10 l of WST solution was added to the cultured media and incubated in the CO_2 incubator for 2 h. Absorbance at 450 nm was measured by spectrophotometer.

Annexin V and PI staining

Annexin V and PI staining was performed by using Annexin V-FITC Apoptosis Detection Kit I according to the manufacturer's instruction. Briefly, cultured cells were trypsinized, washed twice with cold PBS, and centrifuged at 3000 rpm for 5 minutes. The cells were resuspended in 500 μL of 1 × binding buffer at a concentration of 5×10^5 cells/mL and 5 μL of Annexin V-FITC and 5 μL of PI were added. The mixture was incubated for 10 minutes at 37°C in the dark and analyzed by flow cytometry.

RT-PCR (reverse transcription-polymerase chain reaction)

Cultured cells were washed with PBS and total RNA was extracted using Trizol reagent as described in the manufacturer's instructions. cDNA was synthesized and subjected to PCR as described previously [7]. Briefly, reaction mixture for PCR included 2 μL cDNA, 10 pmole of forward and reverse primers, 2 μL of 10 × PCR buffer, 0.2 mM deoxynucleotide triphosphate, and 0.25 U *Taq* DNA polymerase (Cosmo-GenetechCo., Seoul, Korea). Cycling conditions are as follows: denaturation at 95°C for 5 min, followed by amplification cycle at 95°C for 30 sec, appropriate annealing temperature for each primer for 30 sec and 72°C for 30 sec, and final extension at 72°C 7 min. Amplified product was separated on 1.5% agarose gel, stained with ethidium bromide (0.5 μg/mL) for 10 min, and the image was taken by GelDoc Image Analyzer (Biorad, Hercules, USA). The size of the product was compared to 100 bp DNA ladder (Fermentas, Burlington, Canada). The PCR primer sequences used in this study are listed in **Table 1**.

Western blotting

Cells were washed with PBS and then lysed at 4°C with lysis buffer containing 1% Triton X-100, protease inhibitor cocktail, and PBS. Lysates were centrifuged and the supernatants were subjected to Westernblot as described previously [8].

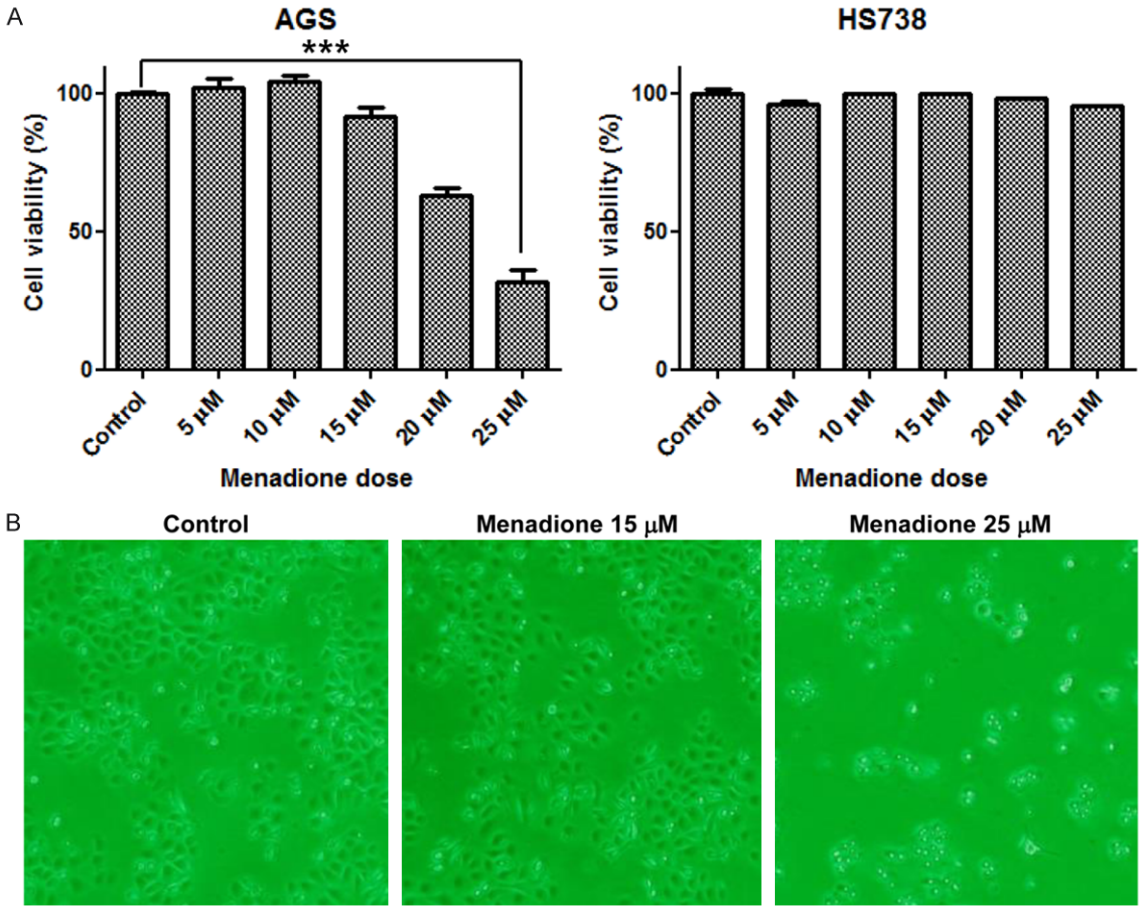

Figure 1. Effect of menadione on cell viability of gastric cancer cell line AGS. A. Either AGS gastric cancer cells or HS738 gastrointestinal cells were treated with indicated dose of menadione (0, 5, 10, 15, 20, 25 μM) for 24 h and cell viability was measured by WST assay. B. AGS cells were treated with indicated dose of menadione for 24 h and images were captured using an inverted microscope (× 400). Data were from three independent experiments and analyzed by unpaired Student's t-test (***$P < 0.001$).

Statistical analysis

Data in the bar graphs are presented as mean ± standard error of mean (SEM). All the statistical analyses were performed using GraphPad Prism 5.02 software (GraphPad Software, San Diego, CA, USA). All the data were analyzed by unpaired Student's t-test and $P < 0.05$ was considered to be statistically significant (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

Results and discussion

Menadione reduces cell viability of gastric cancer cell line but not in non-cancerous cells

The effect of menadione on cell viability in gastric cancer cells was examined using the human gastric adenocarcinoma cell lines AGS. HS738, a non-cancerous gastrointestinal cell line, was also exposed to the same condition and compared as a control group. In a WST assay, cell viability of AGS decreased in a menadione dose-dependent manner, whereas no significant decrease of cell viability was observed on HS738 (**Figure 1A**). Cell viability of AGS decreased to 63% and 32% by 20 μM and 25 μM of menadione treatment respectively and the results were statistically significant ($P = 0.0001$ and $P = 0.0002$ respectively) (**Figure 1A**). These results suggest that menadione effectively inhibits cell viability of gastric cancer cells but not that of non-cancerous cells.

In addition, increased concentrations of menadione caused gradually atrophied cell morphology as well as declined cell count (**Figure 1B**). By microscopic examination, we confirmed that

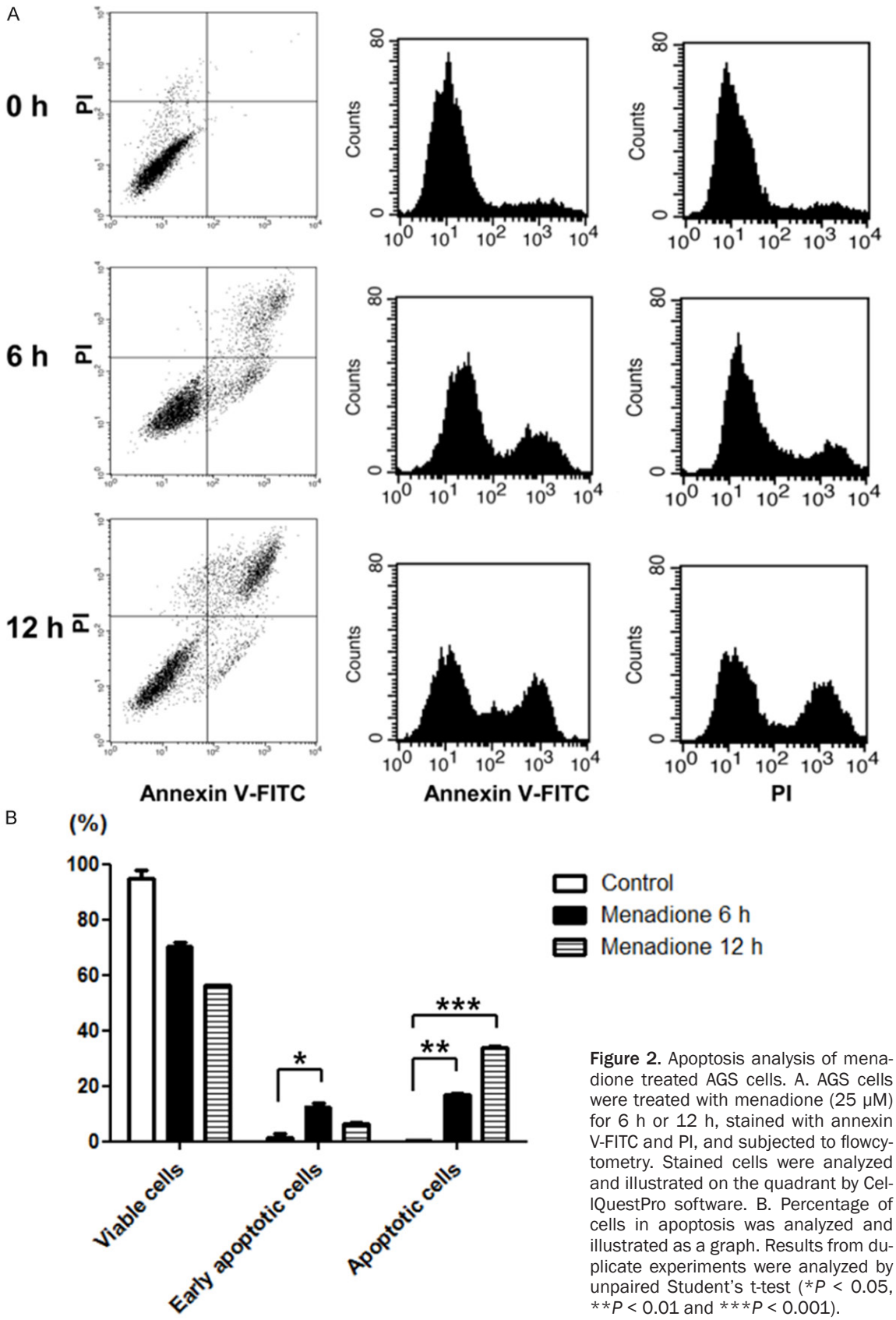

Figure 2. Apoptosis analysis of menadione treated AGS cells. A. AGS cells were treated with menadione (25 μM) for 6 h or 12 h, stained with annexin V-FITC and PI, and subjected to flowcytometry. Stained cells were analyzed and illustrated on the quadrant by CellQuestPro software. B. Percentage of cells in apoptosis was analyzed and illustrated as a graph. Results from duplicate experiments were analyzed by unpaired Student's t-test (*P < 0.05, **P < 0.01 and ***P < 0.001).

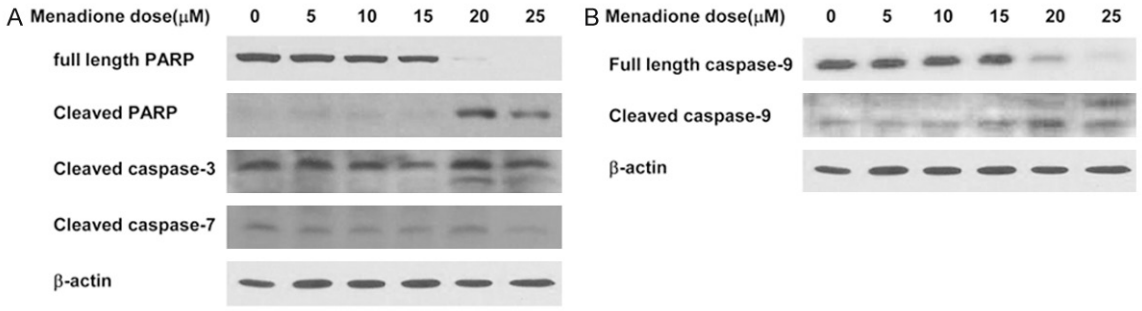

Figure 3. Apoptotic cascade involved in the menadione induced apoptosis of AGS cells. AGS cells were treated with menadione for 6 h and the cell lysates were subjected to Westernblot. A. Westernblot of PARP and effecter caspases. B. Westernblot of initiator caspases.

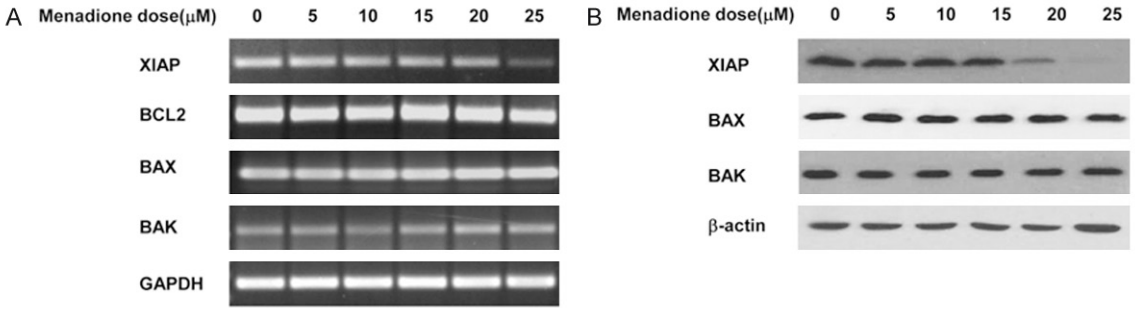

Figure 4. Regulatory molecules associated with the menadione induced apoptosis of AGS cells. AGS cells were treated with indicated doses of menadione for 6 h. A. RNA was harvested and subjected to RT-PCR. B. Cell lysates were subjected to Westernblot.

decrease in MTT reduction was parallel to the morphological changes which are commonly accompanied by apoptotic cell death.

Menadione induces apoptosis via intrinsic caspase cascade in AGS cells

According to the previous reports, menadione has been known to inhibit various types of cancer cells and apoptosis was a major underlying mechanism for the decrease of cell viability of the cancer cells [4-6]. Therefore, we investigated whether decreased cell viability was caused by apoptosis in the current study. AGS cells were treated with 25 μM of menadione and incubated for 6 h or 12 h. To investigate the presence of apoptosis, the cultured cells were then stained with annexin V-FITC and PI and analyzed by flowcytometry. In the results, percentage of cells in early apoptosis and late apoptosis increased to 12.6 % (P = 0.0231) and 16.6 % (P = 0.0014) after 6 h of menadione treatment (**Figure 2A** and **2B**). Then early apoptosis reduced to 6.5 % (P = 0.0645) and late apoptosis further increased to 33.87 % (P < 0.0001) in 12 h of menadione treatment (**Figure 2A** and **2B**). Our results showed that menadione treatment induces apoptosis in AGS gastric cancer cells (**Figure 2A** and **2B**). In particular, early apoptosis was observed in 6 h followed by progression to the late apoptosis in 12 h post-menadione treatment (**Figure 2A** and **2B**).

We confirmed induction of apoptosis after menadione treatment by Westernblot analysis of PARP and caspases. As described above, induction of apoptosis was observed in 6 h according to the flowcytometry results. Thus, we treated varying doses of menadione for 6 h to AGS cells and investigated cleavage of PARP and activation of caspases. In the Westernblot results, we found that full-length form of PARP decreased comparable to the increased cleaved form (**Figure 3A**). The normal function of PARP is repair of DNA breakage [9]. When it is cleaved and inactivated, however, PARP no longer functions and subsequently leads to apop-

tosis of the cell [9]. Cleavage of PARP is accomplished by effecter caspases which involve caspase-3 and caspase-7 [10]. In our results, caspase-3 was activated by menadionetreatment in AGS cells (**Figure 3A**). Furthermore, we also found that caspase-9 was activated by menadione treatment (**Figure 3B**). Caspase-9 is an initiator caspase which is responsible for activation of effecter caspases and it is a key molecule involved in the intrinsic apoptotic cascade [10, 11]. These results indicate that menadione induces apoptosis in gastric cancer cells mediated by caspase-9 and caspase-3 dependent caspase cascade.

Menadione downregulates XIAP expression consequently inducing apoptosis in AGS cells

Activation of apoptotic cascade is regulated by numerous pro-apoptotic and anti-apoptotic molecules [12]. Bcl-2 family proteins are one of the classical regulatory molecules of apoptosis, and there are at least 15 BCL-2 family members in mammals which include both pro-apoptotic and anti-apoptotic proteins [12, 13]. BCL-2 is an anti-apoptotic protein and BCL-2-associated X protein (BAX) and BCL-2-associated killer (BAK) are typical pro-apoptotic BCL-2 family proteins both of which are normally sufficient for cell death [12, 13]. Moreover, there is another family of proteins named inhibitors of apoptosis protein (IAP) which plays a critical role in the regulation of apoptosis by inhibiting function of caspases [12, 14]. XIAP is one of the IAPs that inhibits various caspases especially caspase-3 and caspase-9 [14, 15]. On the basis of these reports, we further investigated upstream regulatory molecules involved in the menadione induced activation of caspases and apoptotic cell death in gastric cancer cells. Among the apoptosis regulatory molecules investigated, we found that mRNA level of XIAP decreased in a menadione dose-dependent manner (**Figure 4A**). However, mRNA expression of Bcl-2 family molecules investigated in this study was unchanged (**Figure 4A**). Moreover, in the Westernblot results, the protein level of XIAP was also decreased in accordance with the mRNA level (**Figure 4B**). From these results, activation of caspase-3 and caspase-9 and the subsequent induction of apoptosis by menadione can be explained because menadione reduces expression of XIAP which is responsible for inhibition of caspase-3 and caspase-9.

In this study, we investigated inhibitory effect of menadione on gastric cancer cells and the inhibitory mechanism. Here we have found that menadione effectively decreased cell viability of gastric cancer cells in the dose that is negligible to non-cancerous gastrointestinal cells. We also have elucidated that decreased cell viability of gastric cancer cells by menadione is mediated by activation of caspase-3 and caspase-9, cleavage of PARP and subsequent induction of apoptosis. Furthermore, decrease of XIAP, which is an inhibitor of apoptosis protein, is involved in the activation of caspases by menadione treatment on gastric cancer cells.

Though the mechanism is yet unclear, it has been reported that naphthoquinones including menadione have suppressive effect on *Helicobacter pylori* which is a class I carcinogen known to induce gastric cancer [16, 17]. Tariq *et al.* reported that menadione shows gastroprotective effect by reducing ulcer on the gastric mucosa [18]. Moreover, it is advantageous that stomach is easily approachable for drugs by oral administration. Therefore, menadione is potentially applicable not only for treatment of gastric cancer but also for other gastric disease, although further studies on detailed mechanism and accumulated reports are necessary as well as *in vivo* evidences.

Acknowledgements

This work was supported by the Graduate School of Yonsei University and the BK 21 plus project, “Field-oriented Graduate Program for Biomedical and Molecular Diagnostics Professionals”, in the Department of Biomedical Laboratory Science, Yonsei University.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jong-Bae Kim, Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University, Wonju 26493, Republic of Korea. Tel: +82-33-760-2423; Fax: +82-33-760-2561; E-mail: kimjb70@yonsei.ac.kr

References

[1] Marchionatti AM, Picotto G, Narvaez CJ, Welsh J and Tolosa de Talamoni NG. Antiproliferative action of menadione and 1,25(OH)2D3 on

breast cancer cells. J Steroid Biochem Mol Biol 2009; 113: 227-232.
[2] Osada S, Tomita H, Tanaka Y, Tokuyama Y, Tanaka H, Sakashita F and Takahashi T. The utility of vitamin K3 (menadione) against pancreatic cancer. Anticancer Res 2008; 28: 45-50.
[3] Tetef M, Margolin K, Ahn C, Akman S, Chow W, Leong L, Morgan RJ Jr, Raschko J, Somlo G and Doroshow JH. Mitomycin C and menadione for the treatment of lung cancer: a phase II trial. Invest New Drugs 1995; 13: 157-162.
[4] Al-Suhaimi E. Molecular mechanisms of leptin and pro-apoptotic signals induced by menadione in HepG2 cells. Saudi J Biol Sci 2014; 21: 582-588.
[5] Kim YJ, Shin YK, Sohn DS and Lee CS. Menadione induces the formation of reactive oxygen species and depletion of GSH-mediated apoptosis and inhibits the FAK-mediated cell invasion. Naunyn Schmiedebergs Arch Pharmacol 2014; 387: 799-809.
[6] Oztopcu-Vatan P, Sayitoglu M, Gunindi M and Inan E. Cytotoxic and apoptotic effects of menadione on rat hepatocellular carcinoma cells. Cytotechnology 2015; 67: 1003-9.
[7] Lee J, Park M, Lee MH, Woo HJ, Kim HW, Yang JY, Eom YB, Kim SH, Yoo C and Kim JB. Development of EBV-encoded small RNA targeted PCR to classify EBV positive diffuse large B-cell lymphoma (DLBCL) of the elderly. Int J Clin Exp Pathol 2015; 8: 7859-7868.
[8] Lee K, Lee MH, Kang YW, Rhee KJ, Kim TU and Kim YS. Parkin induces apoptotic cell death in TNF-alpha-treated cervical cancer cells. BMB Rep 2012; 45: 526-531.
[9] Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE and Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res 1993; 53: 3976-3985.
[10] Nunez G, Benedict MA, Hu Y and Inohara N. Caspases: the proteases of the apoptotic pathway. Oncogene 1998; 17: 3237-3245.
[11] Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES and Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 1997; 91: 479-489.
[12] Igney FH and Krammer PH. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer 2002; 2: 277-288.
[13] Li MX and Dewson G. Mitochondria and apoptosis: emerging concepts. F1000Prime Rep 2015; 7: 42.
[14] de Almagro MC and Vucic D. The inhibitor of apoptosis (IAP) proteins are critical regulators of signaling pathways and targets for anti-cancer therapy. Exp Oncol 2012; 34: 200-211.
[15] Shiozaki EN, Chai J, Rigotti DJ, Riedl SJ, Li P, Srinivasula SM, Alnemri ES, Fairman R and Shi Y. Mechanism of XIAP-mediated inhibition of caspase-9. Mol Cell 2003; 11: 519-527.
[16] Inatsu S, Ohsaki A and Nagata K. Idebenone acts against growth of Helicobacter pylori by inhibiting its respiration. Antimicrob Agents Chemother 2006; 50: 2237-2239.
[17] Romero-Gallo J, Harris EJ, Krishna U, Washington MK, Perez-Perez GI and Peek RM, Jr. Effect of Helicobacter pylori eradication on gastric carcinogenesis. Lab Invest 2008; 88: 328-336.
[18] Tariq M and Al Moutaery A. Menadione protects gastric mucosa against ethanol-induced ulcers. Exp Toxicol Pathol 2005; 56: 393-399.