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Menadione Induced Apoptosis in MKN45 Cells via Down-regulation of Survivin

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Menadione의 Survivin 하향 조절을 통한 MKN45 세포의 세포사멸 유도 효과

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Menadione is known as an anti-tumor factor. Many studies have reported the potential anti-cancer role of menadione against a range of cancer cell lines. In this study, the anti-cancer effects of menadione and the underlying molecular signaling involved in apoptosis was investigated in gastric cancer cell lines. The menadione treatment decreased the cell viability of MKN45 gastric cancer cells. The decreased cell viability was attributed to the induction of apoptosis, which was confirmed by the results indicating the activation of caspase-3 and -7 and the cleavage of PARP in Western blotting. The upstream regulatory molecules involved in apoptosis were investigated further and it was discovered that menadione reduced the expression of survivin, an inhibitor of upstream apoptosis proteins. In addition, a transcription factor β-catenin, which is known to regulate survivin expression, was down-regulated by menadione. A previous report showed that menadione inhibited XIAP expression to induce apoptosis and induced G2/M cell cycle arrest in AGS cells. This study elucidated another inhibitory mechanism of menadione against gastric cancer cells in a different cell line. Although further studies will be needed, the inhibitory mechanism demonstrated in this study will help better understand the anti-cancer effects of menadione.

Key words: Apoptosis, β-catenin, Menadione, MKN45 cells, Survivin

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INTRODUCTION

Naphthoquinones are widespread in nature and synthetic forms of naphthoquinones also exist. Menadione is a synthetic form of naphthoquinones which is also called vitamin K3. As a vitamin K, menadione can play its role for maintenance of blood clotting and bone formation [1, 2]. There have been various reports suggesting that menadione induces apoptosis in cancer cells. Induction of apoptotic cell death by menadione has been reported on

several types of cancer cells including lung cancer, breast cancer, hepatocellular carcinoma, pancreatic cancer and ovarian cancers [1, 3-6]. Menadione induced apoptotic cell death by activation of caspase-3 on hepatocellular carcinoma cells [1]. Activation of caspase-3 and cleavage of PARP by menadione was also observed in pancreatic cancer according to Osadaet al [4]. In ovarian cancer cells, menadione decreased Bid, Bcl-2, Bcl-xL, and survivin protein levels; increased Bax levels; increased release of cytochrome c; activated of caspases (-8, -9, and -3); and increased cleavage of PARP-1 [3].

Activation of apoptotic cascade is regulated by numerous pro-apoptotic and anti-apoptotic molecules [7]. 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 [7, 8]. 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[7,8]. 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 [7, 9].

Survivin is a member of IAP family proteins and it is also called baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5) [10]. This protein is produced by the *BIRC5* gene in human [10]. In contrast to the structure of other IAP family proteins this protein is lack of C-terminal RING finger domain, though it possesses a BIR domain [11, 12]. The survivin protein functions to inhibit apoptotic pathway and negatively regulates apoptosis of the cells [13-15]. Survivin interacts with caspase-3 and 7, an effector caspases of apoptotic pathway [13-15]. Survivin expression is regulated by the cell cycle and is highly expressed in the G2/M phase of the cell cycle to support the cell division [12]. This cell cycle-dependent expression of survivin is due to the cell cycle-dependent element in the promoter of survivin gene that typically present in genes expressed in G2/M phase such as cyclin A and cyclin B [12, 16]. During G2/M phase of the cell cycle, survivin localizes to the mitotic spindle and may play a contributing role in regulating mitosis [12, 17].

This study was conducted to evaluate the anticancer effect of menadione and to demonstrate the molecular signaling involved in apoptosis in human gastric cancer cell lines.

MATERIALS AND METHODS

1. Materials

RPMI 1640 medium, DMEM medium, fetal bovine serum (FBS), streptomycin-penicillin, and trypsin-EDTA were obtained from BRL Life Technologies (Grand Island, NY, USA). EZ-Cytox cell viability assay kit was purchased from Daeil Lab Service (Seoul, Korea). Trizol reagent, random hexamer, and 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, PARP, β-catenin and survivin were purchased from Cell Signaling Technology (Danvers, MA, USA) and β-actin antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2. Cell culture

MKN45 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and streptomycin-penicillin (100 μg/mL and 100 IU/mL). AGS cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and streptomycin-penicillin (100 μg/mL and 100 IU/mL). All the cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. RPMI 1640 medium, DMEM medium, fetal bovine serum (FBS), streptomycin-penicillin, and trypsin-EDTA were obtained from BRL Life Technologies (Grand Island, NY, USA).

3. WST cell viability assay using EZ-Cytox

To measure cell viability, MKN45 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 water

soluble tetrazolium salt (WST) assay by using EZ-Cytox cell viability assay kit (Daeil Lab Service, Seoul, Korea) [18]. Ten μL 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.

4. RT-PCR (reverse transcription-polymerase chain reaction)

To perform RT-PCR RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as described in the manufacturer's instructions. cDNA was synthesized by reverse transcription with 2 μg of total RNA, 0.25 μg of random hexamer (Invitrogen) and 200 U of MMLV-RT (Invitrogen) for 50 min at 37°C and 15 min at 70°C [18]. Subsequent PCR amplification using 0.25 U of *Taq* polymerase was performed in a thermocycler using specific primers [18]. The PCR primer sequences used in this study are as follows: survivin forward 5'-AGCCCTTTCTCAAGGACCAC-3' and reverse 5'-GCACTTTCTTCGCAGTTTCC-3', β-catenin forward 5'-TTGAAGTATACCATACAACTG-3' and reverse 5'-GCAGCATCAAACTGTGTAGAT-3', and GAPDH forward 5'-CGGGAAGCTTGTCATCAA TGG-3' and reverse 5'-GGCAGTGATGGCATGGACTG-3'.

5. Western blotting

Proteins were collected from cells and subjected to Western blotting. MKN45 or AGS cells were washed with PBS and then lysed at 4°C with lysis buffer containing 1% Triton X-100 and protease inhibitor cocktail. The cell lysates were then centrifuged and the supernatants were collected. The proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with optimal concentrations of primary antibody at 4°C overnight and then incubated with the appropriate secondary antibody for 2 h at room temperature. The immune-labeled proteins were visualized using ECL. Antibodies to detect caspase-3, caspase-7, PARP, survivin and β-catenin were purchased from Cell Signaling Technology (Danvers, MA, USA) and β-actin antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

RESULTS

1. Menadione reduces cell viability of MKN45 gastric cancer cell line

The inhibitory effect of menadione on the gastric cancer cells was evaluated. We treated menadione to gastric cancer cell line and assessed changes of cell viability. MKN45 was a gastric cancer cell line used in this study. Various concentrations of menadione (0~30 μM) was treated to MKN45 cells for 24 h and cell viability was measured by WST assay. In our result, cell viability of MKN45 decreased in a menadione dose-dependent manner (Figure 1A). Cell viability of MKN45 decreased to 53.5% and 25.9% by 25 μM and 30 μM of menadione treatment, respectively (Figure 1A). In addition, microscopic examination also has shown that menadione

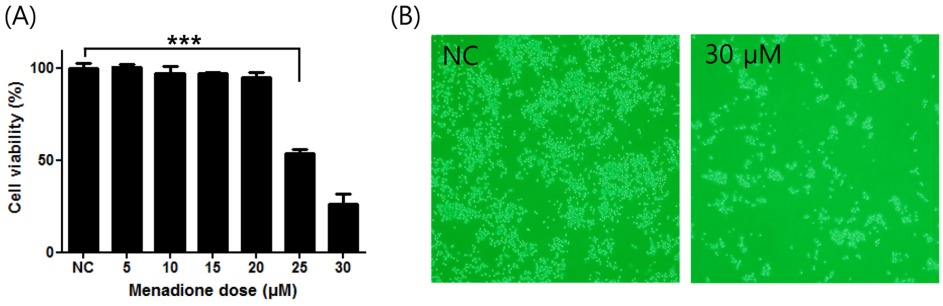

Figure 1. Inhibitory effect of menadione on cell viability of MKN45 gastric cancer cell line. (A) MKN45 gastric cancer cells were treated with indicated dose of menadione (0, 10, 15, 20, 25, and 30 μM) for 24 h and cell viability was measured by WST assay. Data were from three independent experiments and analyzed by unpaired Student's *t*-test (***$P<0.001$). (B) MKN45 cells were treated with or without 30 μM of menadione for 24 h and images were captured using an inverted microscope (×400).

treatment decreases cell confluency (Figure 1B). These results concordantly indicate the inhibitory effects of menadione on cell viability of gastric cancer cells.

2. Menadione induces apoptosis via activation of caspases in MKN45 cells

We next investigated whether menadione decreased cell viability of MKN45 cells via induction of apoptosis. MKN45 cells were treated with menadione and the cultured cells were then subjected to Western blotting analysis of PARP and caspases to investigate the presence of apoptosis. In the Western blotting results, it was found that cleaved form of PARP was increased in a menadione dose-dependent manner (Figure 2). Increased amount of cleaved-PARP is a noted indicator of apoptosis, and the cleavage of PARP protein is conducted by so-called effector caspases such as caspase-3 and caspase-7. Our results also showed that menadione treatment activated both caspase-3 and caspase-7 in MKN45 cells (Figure 2). These results support the idea that menadione reduced cell viability of gastric cancer cells via activation of caspases and induction of apoptosis.

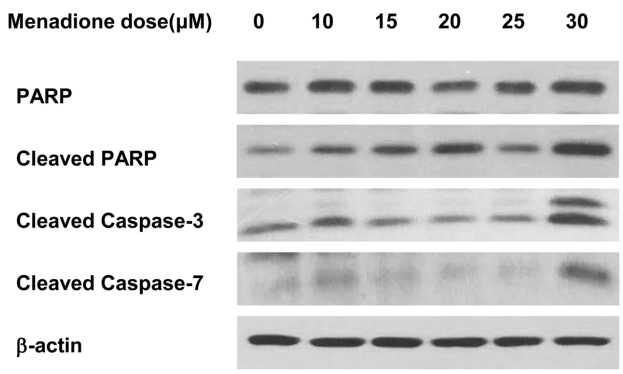

Figure 2. Activation of apoptotic cascade during menadione induced apoptosis of MKN45 cells. MKN45 cells were treated with indicated dose of menadione (0, 10, 15, 20, 25, and 30 μM) for 12 h and the cell lysates were subjected to Western blotting to detect cleavage of PARP, activation of caspase-3 and 7.

3. Menadione down-regulates survivin expression consequently inducing apoptosis in gastric cancer cells

Various molecules are associated with the regulation of caspase activity, and survivin is one of those regulators that inhibit caspase activation. We conducted RT-PCR and Westernblotting to elucidate the upstream regulatory molecule associated with the apoptosis induced by menadione treatment in MKN45 cells. We found that menadione reduced expression of survivin both in mRNA and protein levels (Figure 3A). Down-regulation of survivin was further confirmed in another gastric cancer cell line AGS. Menadione treatment also reduced both mRNA and protein levels of survivin in AGS cells (Figure 3B). In our previous report, we showed that menadione inhibited XIAP expression to induce apoptosis in AGS cells [18]. We also found expression of XIAP was down-regulated by menadione in MKN45 cells as it was shown in our previous report (data not shown). Collectively, reduced expression of XIAP and survivin by menadione treatment explains how it activated caspases and the subsequently induced apoptosis in MKN45 cells. TCF/β-catenin transcription factor was reported to regulate

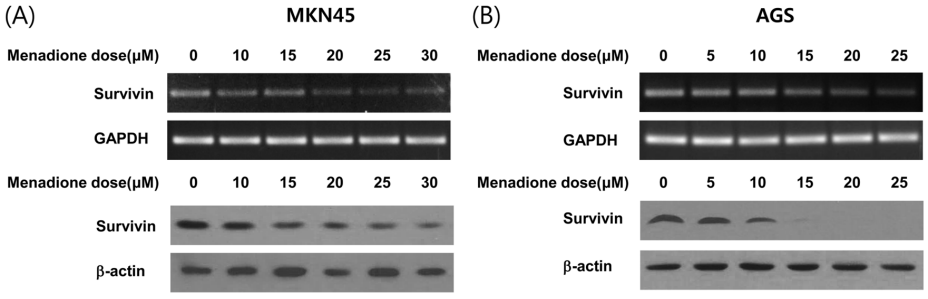

Figure 3. Down-regulation of survivin during menadione induced apoptosis of gastric cancer cell lines. (A) MKN45 cells were treated with indicated doses of menadione (0, 10, 15, 20, 25, and 30 μM) for 12 h. RNA was harvested and subjected to RT-PCR to detect survivin mRNA level (upper figure). Cell lysates were collected and subjected to Western blotting to detect survivin protein level (lower figure). (B) AGS cells were treated with indicated doses of menadione (0, 5, 10, 15, 20, and 25 μM) for 12 h. Cells were harvested and subjected to RT-PCR (upper figure) and Western blotting (lower figure) to detect survivin mRNA and protein level.

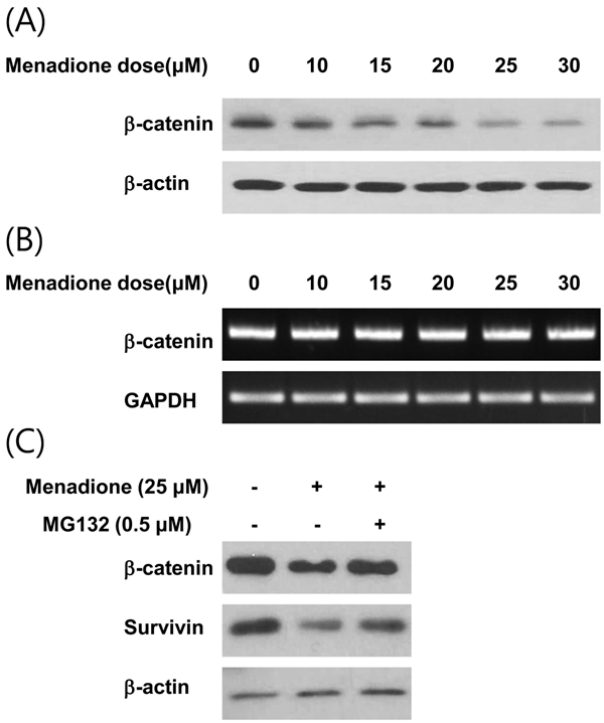

Figure 4. Down-regulation of β-catenin via proteasome-mediated pathway during menadione-induced apoptosis. (A) MKN45 cells were treated with indicated doses of menadione (0, 10, 15, 20, 25, and 30 μM) for 12 h. Cell lysates were collected and subjected to Western blotting to detect β-catenin protein level. (B) Cells were treated as in (A) and subjected to RT-PCR to detect mRNA level of β-catenin. (C) Menadione (25 μM)-treated cells were treated with or without MG132 (0.5 μM) for 12 h. Cell lysates were collected and subjected to Western blotting to detect β-catenin protein level.

survivin promoter activity. Thus we investigated protein level of β-catenin and found that menadione treatment reduced β-catenin protein level in MKN45 cells (Figure 4A). However, menadione treatment did not changed mRNA level of β-catenin (Figure 4B). To elucidate whether menadione down-regulated β-catenin via proteasome mediated pathway, a proteasome inhibitor MG132 was treated to the cells. The decrease of β-catenin by menadione was partially alleviated in the cells treated with MG132, and survivin protein level was also partially recovered in concordance (Figure 4C). The result showed that proteasome mediated degradation is associated with the decrease of β-catenin by menadione treatment.

DISCUSSION

Gastric cancer accounts for 8.5% of cancer worldwide and 35.4% of the gastric cancer occurred in Eastern Asia [19, 20]. Prevalent infection of *Helicobacter pylori*, a class I carcinogen, may have contributed to the high incidence of gastric cancer in Eastern Asia [21]. Park et al. described inhibitory effect of naphthoquinones such as menadione against *H. pylori*. Tariq et al. suggested menadione has gastroprotective effect and reduces gastric ulcer [22]. These reports collectively show the potential availability of menadione for prevention of gastric disease. In this study, inhibitory effect of menadione on MKN45 cells and the inhibitory mechanism have been investigated. Here it has been found that menadione decreased cell viability of MKN45 cells. Decreased cell viability of gastric cancer cells by menadione was mediated by activation of caspase-3, caspase-7, and cleavage of PARP and subsequent induction of apoptosis. Decrease of survivin, which are inhibitor of apoptosis proteins, were involved in the activation of caspases by menadione treatment on gastric cancer cells.

We have elucidated β-catenin was associated with the down-regulation of survivin by menadione. However, various mechanisms have been reported to regulate survivin expression. The expression of survivin was dependent on the SP1 transcription factor [23]. It was also reported that the expression of survivin is inhibited by p53-mediated transcriptional repression [24]. Persistent activation of Stat3 was reported to induce survivin expression in breast cancer cells [25]. Thus, the other molecular mechanisms may contribute to the down-regulation of survivin in gastric cancer cells treated with menadione.

In addition, we have previously reported that menadione inhibits gastric cancer cell lines by inducing G2/M cell cycle arrest [26]. Survivin expression is regulated by the cell cycle and is highly expressed in the G2/M phase of the cell cycle to support the cell division [12]. This cell cycle-dependent expression of survivin is due to the cell cycle-dependent element in the promoter of survivin gene

that typically present in genes expressed in G2/M phase such as cyclin A and cyclin B [12, 16]. During G2/M phase of the cell cycle, survivin localizes to the mitotic spindle and may play a contributing role in regulating mitosis [12, 17]. Therefore, we presume that inhibited expression of survivin may also have contributed to the G2/M cell cycle arrest in gastric cancer cells induced by menadione treatment.

The inhibitory effects of menadione on gastric cancer cells and the inhibitory mechanism demonstrated in this study suggests the potential availability of menadione for treatment of gastric cancer. However, further studies still seem to be necessary to completely understand the inhibitory mechanisms and physiological availability *in vivo*.

요 약

Menadione은 종양 억제 물질로 알려진 바 있다. 현재 많은 연구에서 다양한 암세포주에 대하여 Menadione의 잠재적인 항암물질로서의 가능성이 보고되었다. 본 연구에서는 Menadione의 항암효과와 세포사멸작용에 연관된 분자신호를 위암세포주에서 확인하였다. Menadione 처리는 위암세포인 MKN45의 세포생존능을 감소시켰다. 감소된 세포생존능은 Western blotting을 통해 caspase-3 과 caspase-7의 활성화와 PARP가 cleavage 된 것을 확인함으로써 세포사멸작용이 유도되었다는 것을 확인했다. 위세포사멸단백질들의 저해제로 작용하는 survivin의 발현을 menadione이 억제한다는 것을 확인함으로써, 세포사멸과정에 포함된 상위조절인자를 확인했다. 우리는 survivin 발현을 조절하는 전사인자로 알려진 β-catenin 또한 menadione에 의해 하향 조절된다는 것을 확인했다. 이전 연구에서 우리는 menadione이 세포사멸유도를 저해는 XIAP의 발현을 억제한다는 것을 확인했으며, menadione이 AGS세포에서 G2/M 세포주기 정체를 유도한다는 것을 확인하였다. 우리는 또 다른 위암세포주인 MKM45 세포에서 menadione의 이전과 다른 항암 기전을 밝혀냈다. 비록 더 자세한 연구가 필요하겠지만, 이 연구를 통해 증명된 억제기전은 menadione에 의한 항암효과를 이해하는 데 도움이 될 것으로 사료된다.

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