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Cortex Mori Extract Induces Cancer Cell Apoptosis Through Inhibition of Microtubule Assembly

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The water extract from the root bark of Cortex Mori (CM, Morus alba L.: Sangbaikpi), a mulberry tree, has been known in Chinese traditional medicine to have antiphlogistic, diuretic, and expectorant properties. In this study, the cytotoxicity of CM against tumor cells and its mechanism was examined. CM exhibited cytotoxic activity on K-562, B380 human leukemia cells and B16 mouse melanoma cells at concentrations of > 1 mg/ml. A DNA fragmentation, PARP cleavage, and nuclear condensation assay showed that those cells exposed to CM underwent apoptosis. The water extract of Scutellarie Radix (SR) was used as a negative control and showed no cytotoxicity in those cells. The flow cytometric profiles of the CM-treated cells were also indicative of apoptosis. However, they did not appear to exert the G1 arrest, which is observed in other tubulin inhibitor agents such as vincristine, taxol. The protein-binding test using Biacore and a microtubule assembly-disassembly assay provided evidence showing that CM bound to the tubulins resulting in a marked inhibition of the assembly, but not the disassembly of microtubules. The possible nonspecific effect of the CM extract could be excluded due to the results using SR, which did not affect the assembly process. Overall, the water extract of CM induces apoptosis of tumor cells by inhibiting microtubule assembly.

Key words : *Cortex Mori*; cytotoxicity; apoptosis; tubulin; microtubule assembly

INTRODUCTION

Cortex Mori (*CM*), commonly known as "Sangbaikpi" in Korea, "Sanbaipi" in China or "Sohakuhi" in Japan, is the root bark of mulberry trees including *Morus alba* L., *Morus bombycis* K., *Morus multicaulis* P., and *Morus lhou* K. It has long been used as an antiphlogistic, diuretic and expectorant (Nomura, 1988). Therefore, this herb has been used for the control of inflammation, diabetes, and bronchial asthma (Nanba, 1981; Kimura and Kimura, 1981). Recent studies have shown that *CM* has many other activities, such as hypotensive activity (Nomura, 1988; Fukutome, 1938; Nomura *et al.*, 1980; Nomura and Fukai, 1980; Fukai *et al.*, 1985), antiviral activity (Ishitsuka *et al.*, 1982), it inhibits cAMP phosphodiesterase activity (Nikaido, 1984), and regulates the arachidonate metabolism in rat platelet homogenates (Kimura *et al.*, 1986). *CM* was recently shown to inhibit histamine release of rat mast cells by blocking calcium uptake (Chai *et al.*, 1999). The antitumor activity of *CM* was also reported by Nomura *et al* (1988) and the cytotoxicity of *CM* against tumor cell lines has been observed (Kim *et al.*, 2000). However, the mechanisms underlying the antitumor activity are unclear. Recent findings showing that *CM* inhibits the morphological change, degranulation and histamine release of mast cells (Chai *et al.*, 1999) exposed to human seminal plasma, prompted us to investigate *CM* as an antimicrotubule agent. In this study, the data showed that *CM* exerts cytotoxicity against tumor cells by inducing apoptosis. Moreover, *CM* binds tubulin to inhibit the microtubule assembly.

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MATERIALS AND METHODS

Preparation of CM extract and cells

CM was purchased from the oriental drug store, Bohwa

Dang (Jeonju, Korea), and a voucher specimen was deposited at the Herbarium of the Chonbuk National University. The water-soluble components were extracted from the dried and powdered roots (4 kg) by boiling for 5 hours in distilled water. This aqueous extract was filtered through a 0.45-mm filter, lyophilized with rotary vacuum evaporator (EYELA, Tokyo Rikakikai Co. Ltd, Japan) to give the *CM* extract (20.73 g) and stored at 4°C. The dried extract was dissolved in a Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% bovine serum albumin) prior to use (100 mg/ml). The water extract of *Scutellarie Radix* (*SR*) was also prepared as above and used as a negative control drug. The human leukemic cell line, K-562 and B380 cells, were maintained in complete RPMI 1640 medium (Life Technologies, Gaithersberg, MD. USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Life Technologies). The B16 mouse melanoma cell line was also maintained in a complete MEM (Life Technologies) medium.

Cytotoxicity assay

The cytotoxicity of *CM* on cancer cells was examined by a MTT assay. Briefly, the cells were plated in a 24 well culture plate and *CM* was added at the indicated concentrations. Two days later, MTT (Sigma, St Louis, MO. USA) was dissolved at 2 mg/ml in sterile PBS, filtered and added at a final concentration of 400 μg/ml. After 4 hours incubation, the formazan crystals were dissolved with 0.04N HCl-isopropanol (0.04 N HCl in isopropanol) and quantified by measuring the absorbance at 570 nm with a reference filter of 630 nm using an automatic ELISA reader (Bio-Rad, Hercules, CA, USA).

DNA fragmentation assay

After treating the cells with *CM* (5 mg/ml) for 24 hours, they were washed with cold PBS and lysed by incubating them in 1 ml of a lysate buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA and Triton X-l00) for 10 min on ice. The lysate was centrifuged at 14000 r.p.m. for 5 min. The supernatant was extracted with phenol: chloroform and the DNA was alcohol precipitated as previously described (Shao *et al.*, 1996). The samples were dissolved in 20 μl of water and incubated at 37°C for 30 min. The DNA samples were analyzed by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

Western blot analysis for PARP cleavage

The proteins were extracted from the *CM*-treated K-562 and B380 cells using a lysis buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tri-HCl (pH 8.0), 1%-NP 40, 1 mM aprotinin, 0.1 mM leupeptin 1 mM pepstatin, and quantified using the bicinchoninic acid protein assay (Pierce, Rockford,IL, USA). A total of 10 μg of protein was electrophoresed in 8% SDS-polyacrylamide gels under reducing conditions and transferred to Hybond-P (Amersham, Arlington, IL, USA) using the Mini-protean II system (Bio-Rad, Hercules, CA, USA). After blocking with 5% skim milk in PBS, the mixture was incubated with rabbit polyclonal anti-PARP (the nuclear enzyme poly(ADP-ribose) polymerase) antibodies (Cell Signaling Tech, MA, USA) at 2 μg/ml dilution in PBS for 1 hour at room temperature. This was followed by one 15 min and two 5 min washes with PBS plus a 0.1% Tween 20 buffer. The membranes were subsequently incubated with anti-rabbit IgG conjugated with horseradish peroxidase at 1 : 3,000 dilution in PBS for 1 hour at room temperature. After the final wash, the immunoreactive bands were detected by enhanced chemiluminescence using Kodak film.

Nuclear condensation

The murine melanoma B 16 cells were incubated in a 6 well plate with or without *CM* (5 mg/ml) for 24 hours. After washing with PBS, the cells were fixed with methyl alcohol/acetic acid (3:1) on a slide and dried. The fixed cells washed with PBS, air dried and stained with a Hoechst 33258 solution (0.1 mg/ml) (Sigma, St. Louis, Mo, USA). Following 10 min of incubation, the cells were washed, air dried and mounted with synthetic mountant (Shandon, Pittsburg, PA, USA). The plates were observed using fluorescence microscopy (Olympus Optical Co., Japan).

Flow cytometry for cell cycle analysis

The cell cycle was examined by flow cytometry with propidium iodide (PI) staining. The K-562 cells were plated at a density of 100,000 cells per 100-mm dish in 10% FBS-RPMI medium. Twenty-four hours later, the cells were treated with *CM* at the indicated concentration for 24 hours. The cells were pooled and fixed in 70% cold ethanol in PBS for at least 30 min on ice. The cells were pooled, washed in PBS, stained with PI (20 μg/ml) and incubated with 20 μg/ml of RNase A for at least 30 min. The samples were analyzed using an EPIC profile analyzer. The histograms showing the total DNA content as Fl 2 vs the cell number are shown.

Biomolecule Interaction (Biacore) assay for Tubulin-binding analysis

Tubulin was coupled to a CM5 chip using the standard amine immobilization protocols. Briefly, the chip was activated with 50 mM N'-hydroxy-succinimide and 200

mM N-(dimethylaminopropyl)-N'-ethylcarbodimide. Five mg tubulin was reconstituted in 1 μl of MES (1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA. 100 μg/ml sucrose. 1 mM DTT, 0.1 mM GTP, 1 μg/ml leupeptin, 1 μg/ml aprotinin, pH. 6.8). The reconstituted tubulin was diluted 1 : 50 in sodium acetate, pH. 4.7. It was then injected at a flow rate of 10 μl/min and coupled to the chip.

For the binding assay, the indicated *CM* extract concentration in 20 μl HBS buffer was injected for 30 sec at a flow rate of 20 μl/ml over two flow cells, one with coupled tubulin and other with BSA.

Inhibition assay of microtubule assembly and disassembly

The purified tubulin prepared from a calf brain was purchased from Sigma and its assembly and disassembly assay was carried out previously (Jiang *et al.*, 1998). Briefly, to inhibit assembly, 100 μl of the tubulin solution (500 μg protein/ml) were mixed gently with 400 μl of a reaction buffer (0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM EDTA, 2.5 M glycerol) and 1mg/ml of *CM* at 37°C. After adding 1 mM GTP (Sigma) to each sample cuvette, the microtubule assembly was monitored by measuring the change in optical density at room temperature at 350 nm every 5 min on a spectrophotometer until the assembly process was complete, usually within 40 min. To inhibit disassembly, 1 mg/ml of *CM* was added to the cuvette with repolymerized microtubules according to the method described above and incubated on melting ice. The changes in optical density were monitored at 350 nm for 30 min until the optical density in the controls returned to the initial value. The SR extract was also examined in parallel as a negative control.

RESULTS

The cytotoxicity of *CM* was determined using a MTT assay. As shown in Fig. 1, *CM* exhibited cytotoxicity

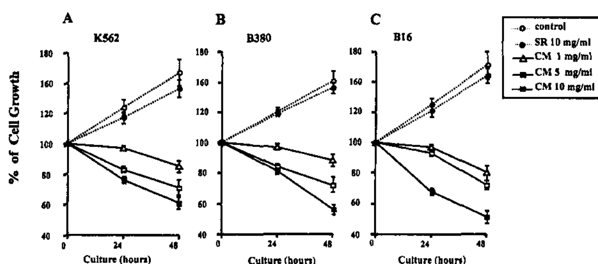

Fig. 1. Inhibition of cell growth by *CM* against tumor cell lines. K-562 (A), B380 (B) and B16 melanoma (C) cells were incubated with 0-10 mg/ml of *CM* for 48 hours and the cytotoxicity was determined using a MTT assay at 24 and 48 hours. The data is presented as a means ± standard deviation of three independent experiments. *CM, Cortex Mori ; SR, Scutellarie Radix*

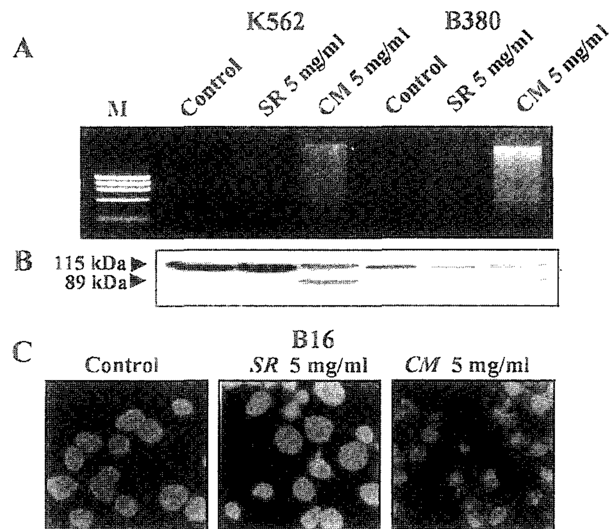

Fig. 2. Induction of apoptosis by *CM* in the K-562, B380 and B16 cells. (A) DNA fragmentation. The cells were incubated with 5 mg/ml of *CM* for 24 hours. DNA was extracted from the harvested cells after treatment with *CM*, analyzed by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining. (B) Cleavage of PARP by *CR* in K-562 cells. The lysates obtained from the cells after treatment with 5 mg/ml of *CM* for 24 hours were analyzed for the apoptosis-specific cleavage of PARP. The intact (116 kDa) and cleaved (85 kDa) forms of PARP were detected by immunoblotting with anti-PARP antibodies. (C) Nuclear condensation analysis in B16 cells. The cells were treated with 5 mg/ml of *CM* for 24 hours and stained with Hoechst 33258 for nuclear staining. The stained cells were examined by fluorescence microscopy. M, a molecular marker (jX174/Hae III) ; *CM, Cortex Mori ; SR, Scutellarie Radix*

against all of three tumor cell lines. The cytotoxicity was detected at > 1 mg/ml and prominently at relatively high concentrations (> 5 mg/ml). In contrast, the negative control, SR, did not show any cytotoxicity at even high concentrations. In order to further understand the *CM*-mediated cytotoxicity, *CM* was examined to determine if it induced internucleosomal degradation of DNA, a characteristic of apoptosis. The DNA extracted from the cells exposed to *CM* showed a laddering phenomenon (Fig. 2A). The nuclear morphological changes were also examined using a DNA-specific fluorescent dye, Hoechst 33258. When the B16 cells were treated with 5 mg/ml of *CM* for 24 hours, they exhibited condensed and fragmented nuclei, which indicative of an apoptotic cell morphology (Fig. 2C). To further confirm apoptotic cell death, the protein levels of a key apoptosis-linked protease, poly(ADP-ribose) polymerase (PARP) were analyzed. PARP, a caspase 3 substrate, is important for maintaining cell viability and appears to be involved in DNA repair in response to environmental stress. PARP cleavage facilitates cellular disassembly and serves as a marker for the cells undergoing apoptosis (Oliver *et al.*, 1998). Proteolysis of the 116 kDa full-length PARP to an 85 kDa

PARP activated fragment was monitored using anti-PARP antibodies. The 85 kDa fragment of the active enzyme was present in the cells treated with 5 mg/ml of *CM* for 24 hours, but not in the non-treated control cells or the *SR*-treated cells (Fig. 2B). This suggests that *CM* induces apoptosis in those cells. However, no apoptotic cells were observed in either the medium alone or with *SR*.

Moreover, apoptosis of the K-562 cells induced by *CM* was confirmed with PI staining and cell cycle analysis. After incubation with *CM*, the apoptotic population was

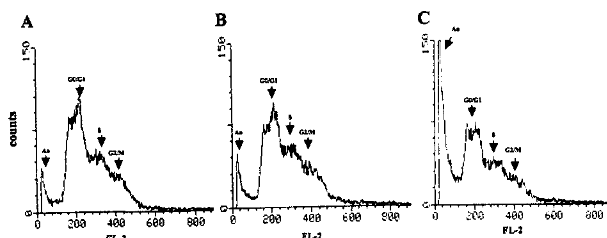

Fig. 3. Cell cycle analysis of K-562 cells after treatment with different concentrations of *CM* for 24 hrs. The harvested tumor cells were fixed and stained with propidium iodide (20 mg/ml) and then analyzed by flow cytometry. Histograms showing the total DNA content, as Fl2 vs cell number, are shown. (A) No treatment with *CR* , (B) Treatment with 5 mg/ml of CM for 24 hours, (C) Treatment with 10 mg/ml of CM for 24 hours.

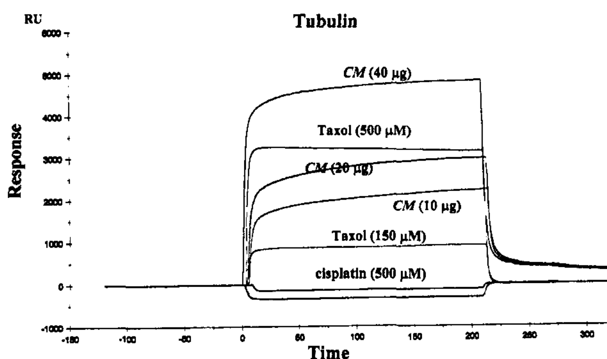

Fig. 4. Binding assay of *CM* to tubulin. *CM* was mixed with tubulin and its binding was analyzed using a Biacore as described in Methods.

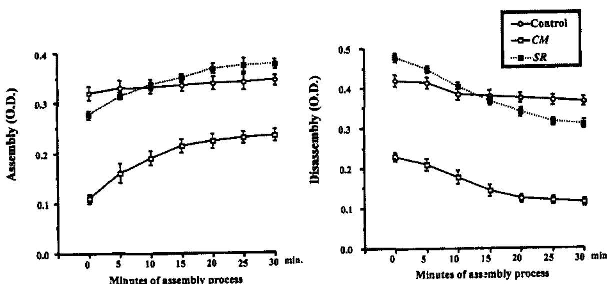

Fig. 5. Inhibition of microtubule assembly but not disassembly by CM. The temperature-dependent tubulin assembly (A) and disassembly (B) was analyzed by determining the O.D. The data is presented as a mean ± standard deviation of three independent experiments. *CM, Cortex Mori ; SR, Scutellarie Radix*

apparent, as shown in Fig. 3. However, the frequencies of the other populations were unaltered. These profiles show that *CM* treatment does not result in the G1 arrest, which is unlike the usual chemotherapeutic agents provoking apoptosis.

CM was analyzed for its binding to tubulin and its inhibitory activity for tubulin assembly or disassembly using Biacore. As shown in Fig. 4, *CM* strongly bound to tubulin in a similar manner as another known tubulin-binder, taxol. Fig 5 also shows the kinetics of microtubule assembly and disassembly, where the control tubulin exhibited a temperature-dependent assembly and disassembly. Tubulin assembly was detected by the increase in the O.D (optical density, 0.107→0.229) for 30 min. The addition of the *CM* extract (100 μg/ml) increased the basal level of the O.D. to 0.320, which resulted in substantial inhibition of tubulin assembly (O.D., 0.320→ 0.354). In contrast, disassembly in the presence of *CM* (O.D., 0.440→0.326) was comparable to the control (0.227→0.108). Other possible nonspecific effects of the *CM* extract could be excluded by the results with SR, which also increased the basal level O.D (0.280). Nonetheless, it did not affect the tubulin assembly (O.D., 0.280 → 0.410) and disassembly (O.D., 0.452 → 0.295).

DISCUSSION

Numerous materials isolated from plants are being investigated for their therapeutic application against many diseases that do not respond to standard therapies, such as cancer, diabetes and hypertension. Mulberry trees are widely cultivated in East Asia and the leaves are used to feed silkworms. Its root bark, *CM* has attracted much interest due to its hypotensive and anti-diabetic activity (Nomura, 1988; Nanba, 1981; Fukutome, 1938 Nomura *et al.*, 1980; Nomura and Fukai, 1980; Fukai *et al.*, 1985). A recent report also showed the cytotoxicity of *CM* against tumor cell lines (Kim *et al.*, 2000), However, its functional mechanisms were not reported. In this study, the induction of apoptosis by *CM* and its active mechanisms was investigated.

The *CM* extract exhibited cytotoxicity at relatively high concentration (5 mg/ml). However, this cytotoxicity was not attributed to physicochemical cell damage, as *SR* extract did not inhibit tumor cell growth even with a higher dose (10 mg/ml). To determine if this significant reduction in tumor cell growth was caused by the apoptosis triggered by *CM*, DNA fragmentation, characteristics of cells undergoing apoptosis, was examined. DNA fragmentation resulting in laddering, PARP cleavage, and nuclear condensation were observed in the CM-treated cells, but not in the non-treated control cells. To further confirm apoptotic cell death, cell cycle analysis of the

tumor cells after treatment with different concentrations of *CM* for 24 hours was examined using a FACScan. In the flow cytometric analysis, there were slightly more cells in the S and G2/M phase in the low concentration *CM*-treated cells than in the non-treated control cells. The proportion of cells in the apoptotic phase (A_0) was significantly higher and that in the S and G2/M phase existed in a sufficient number in the cells treated with high *CM* concentration. General chemotherapeutic agents that induced apoptosis mediated G1 arrest of the cell cycle. However, *CM* induced apoptosis occurred after the S and G2/M arrest of the cell cycle, and the extracts did not exert G1 arrest. These flow cytometric profiles had the same characteristics of other tubulin inhibitor agents such as vinblastine, taxol. Consequently, the tubulin-binding assay by biomolecule interaction analysis (Biacore assay) and the inhibition assay of the microtubule assembly and disassembly were performed to stimulate interest in determining the induction of the apoptotic mechanism of the *CM* extracts. The final data with *CM* and *SR* as a negative control drug showed that *CM* binds to tubulin and subsequently inhibits microtubule assembly, which did not result from the nonspecific masking of tubulin-tubulin contact by solid particles existing in the *CM* extract.

Microtubules are important for interphase functions such as maintaining cell shape, cell motility, and intracellular transport as well as the movement of chromosomes during mitosis (Dustin. 1980). Therefore, tubulin-binding agents may affect the cells during both the interphase and mitotic cell cycle phases. These reagents have two tactics for the mitotic blocking activity by interfering with the exchange of the tubulin subunits between the microtubules and the free tubulin pool in the cytoplasm (Alberts *et al.*, 1994). Antimicrotubule agents such as vincristine and vinblastine induce microtubule disassembly (Rowinsky and Donehower, 1991). In contrast, taxol promotes the assembly of microtubules and stabilizes the tubulin polymers by preventing their depolymerization (shiff *et al.*, 1979; Manfredi and Horwitz, 1984). These agents are large dimeric natural complexes from plants or synthetic variants. In this study, *CM* was demonstrated to bind to tubulin and inhibit its polymerization. This suggests it may be a useful candidate as a chemotherapeutic agent against cancer as a tubulin inhibitor.

The active components in *CM* have not been clarified. Phenolic constituents, flavonoids and related compounds have been isolated from *CM* by Nomura *et al.* (1980), and Kimura *et al.* (1986), respectively. Hikino *et al.* (1985) and Kim *et al.* (1999) have also isolated moran A; a glycoprotein known for its hypoglycemic activity. This study and other investigations (Shin *et al.*, 1998) suggest the possible application of *CM* to treat many diseases.

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