SPANDIDOS PUBLICATIONS

Induction of apoptosis by rhapontin having stilbene moiety, a component of rhubarb (*Rheum officinale Baillon*) in human stomach cancer KATO III cells

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Abstract. We have investigated the effects of rhapontin on proliferation and DNA of human stomach cancer KATO III cells. Growth inhibition and induction of apoptosis by rhapontin were observed in the KATO III cells. Morphological change showing apoptotic bodies was observed in the KATO III cells treated with rhapontin. The fragmentation of DNA by rhapontin to oligonucleosomal-sized fragments that is a characteristic of apoptosis was observed to be concentration- and time-dependent in the KATO III cells. N-acetyl-L-cysteine, an antioxidant, suppressed the DNA fragmentation caused by rhapontin. On the other hand, it was found that resveratrol having stilbene moiety as well as rhapontin induced apoptosis in the KATO III cells. So, it is considered that stilbene moiety in the molecule is essential for the induction of apoptosis. The data of the present study show that the suppression of KATO III cell-growth by rhapontin results from the induction of apoptosis by the compound, and that active oxygen is involved in the inductions of apoptosis caused by rhapontin in the KATO III cells.

Introduction

Rhubarb (*Rheum officinale Baillon*) is a hardy perennial plants, growing mainly at the higher land of more than 3000 m in China. Rootstock of this plant is traditionally used as a purgative. Many compounds such as physcion, sennidine A and B, sennoside A and B, chrysophanol, emodin, anthrone, aloe-emodin, rhein and rhapontin were reported to be isolated from rhubarb (1).

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Thus far we have purified several compounds extracted from plants and evaluated the antitumour activity of those compounds (2-7). The efficiency of the antitumour compounds seems to be related to the propensity of tumour cells to respond to these compounds by apoptosis. Considerable attention has been focused on the sequence of events referred to as apoptosis, and the role of this process in mediating the lethal effects of antineoplastic agents in leukemic cells (8). Apoptosis is a highly regulated process that is characterized by cell shrinkage, membrane blebbing, chromatin condensation and formation of a DNA ladder with multiple fragments of 180-200 bp caused by inter-nucleosomal DNA cleavage (9).

In previous studies we demonstrated the induction of apoptosis by phytol (2), sesamin (3), pheophorbide *a* (4), diol- and triol-types of phytol (5), lupeol (6) and procyanidin (7) in cultured human lymphoid leukemia and stomach cancer cells.

In this study, we demonstrate for the first time that rhapontin, a component of rhubarb (*Rheum officinale Baillon*), induces apoptosis resulting in suppression of proliferation of human stomach cancer KATO III cells, and that stilbene moiety in the molecule is essential for the induction of apoptosis.

Materials and methods

Chemicals. Rhapontin and resveratrol were purchased from Sigma Chemical Co., St. Louis, MO, USA. Chemical structure of rhapontin used in this study is shown in Fig. 1. RPMI-1640 medium and fetal bovine serum were purchased from Gibco Laboratories, Grand Island, NY, USA. Lymphocyte separation medium (LSM) was purchased from ICN Biomedicals Inc., Aurora, OH, USA. All other reagents were of the highest grade.

Cell culture. Human stomach cancer KATO III cells were originally provided by ATCC and obtained from the Health Science Research Resources Bank (HSRRS), Osaka, Japan. KATO III cells were grown in 45% RPMI-1640 medium with 45% Eagle's minimal essential medium containing 10% heat-inactivated fetal bovine serum, penicillin G (50 IU/ml) and streptomycin (50 μg/ml). These cells were cultivated at 37˚C

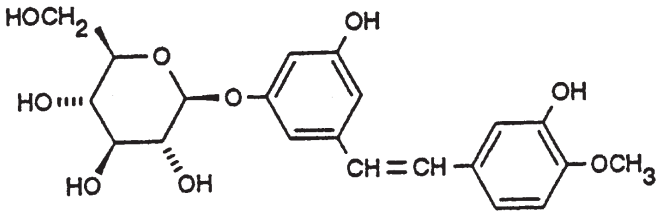

Figure 1. Chemical structure of rhapontin.

under humidified 95% air - 5% CO_2 atmosphere, and passaged every 4 days. Mycoplasma testing was routinely negative.

Exponentially growing human KATO III cells were placed in triplicate at 4 to $5x10^5$ cells/ml in the culture flask and cultivated in the presence of a vehicle (50% ethanol) or rhapontin. After cultivation for 1-3 days, the viable cell number was evaluated by using the Trypan blue dye exclusion method.

For preparation of normal human lymphocyte cells, 3 ml of lymphocyte separation medium (LSM) was aseptically transferred to a centrifuge tube and diluted blood (heparinized blood:physiological saline = 1:1) was layered over the LSM in the tube. The tube was centrifuged at 400 x g at room temperature for 20 min. The top layer of clear plasma was removed, and the lymphocyte layer was transferred to a new centrifuge tube. An equal volume of phosphate-buffered saline (pH 7.5) was added to the lymphocyte layer in the tube and centrifuged for 10 min at room temperature at 260 x g. After the centrifugation, the precipitated lymphocyte was washed with phosphate-buffered saline (pH 7.5), and suspended in RPMI-1640 medium containing 10% FCS and 2% phytohemaglutinin-M (Gibco Laboratories). The obtained normal human lymphocyte cells were cultivated in the presence of a vehicle or rhapontin for 3 days.

Microscopic observation of morphological change of KATO III cells. Exponentially growing KATO III cells were placed at the initial density of 4 to $5x10^5$ cells/ml. After cultivation for 3 days in the presence of a vehicle (50% ethanol), or rhapontin, the morphology of the cells was examined by a epifluorescence microscope (Carl Zeiss, Jena, Germany) with a cooled CCD camera digital imaging system (PxL 1400, Phometrics, AZ, USA) and Fuji pictography 3000 as described by Okumura *et al* (10).

Assay for DNA fragmentation. Exponentially growing human KATO III cells were placed at the initial density of 4 to $5x10^5$ cells/ml in the culture flask. After cultivation in the presence of a vehicle (50% ethanol), rhapontin or resveratrol for 1-3 days, the cells were pelleted by slow centrifugation. DNA was isolated from the cell pellets as described previously (11). Equivalent amounts of DNA (2 μg) were put into wells of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid (pH 7.5) containing 2 mM EDTA.

Effects of N-acetyl-L-cysteine, an antioxidant, on the DNA fragmentation by rhapontin. Exponentially growing KATO III cells were plated at 4 to $5x10^5$ cells/ml. After pre-incubation for 2 h with N-acetyl-L-cysteine, a known antioxidant, the cells were added with rhapontin or resveratrol, and cultured for 3 days. After cultivation, the cells were harvested and DNA was isolated from the cell pellets. Equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed.

Table I. Effects of rhapontin on the growth of KATO III cells

	Concentration (μM)	Inhibition (%)
Vehicle (50% ethanol)	0	0
rhapontin	50	29.3
	100	56.8
	200	98.6

KATO III cells were exposed to rhapontin at the indicated concentrations for 3 days before the viable cell number was estimated by trypan blue dye exclusion method. Each value is the mean of triplicate experiments.

Results and Discussion

Effect of rhapontin on the growth of KATO III cells. The effect of rhapontin on the proliferation of human KATO III cells were studied. As shown in Table I, the proliferation of KATO III cells was inhibited significantly by the compound in a dose-dependent manner.

Induction of apoptosis by rhapontin. The significant growth-inhibitory activity of rhapontin led us to investigate whether part of the antitumour effect of rhapontin was a result of apoptosis induction. Morphological change showing apoptotic bodies and fragmentation of genomic DNA into oligonucleosomal-sized fragments are characteristics of the occurrence of apoptosis. The morphology of the treated KATO III cells shows apoptotic bodies after 3 days of treatment with 200 μM rhapontin (Fig. 2). Rhapontin was specific for the induction of apoptosis in KATO III cells, because other compounds from rhubarb such as physcion, sennidine A and B, sennoside A and B, chrysophanol, emodin, anthrone and rhein did not induce apoptosis. In the present study, apoptosis by rhapontin in the KATO III cells was observed for the first time.

Dose- and time-dependence of apoptosis by rhapontin. As shown in Fig. 3, oligonucleosomal-sized fragments were observed in the KATO III cells treated with rhapontin. Induction of apoptosis by rhapontin in the KATO III cells occurred both dose- (from 50 to 200 μM) (Fig. 3) and time-dependently (from 1 to 3 days) (Fig. 4). Based on these findings it is considered that growth inhibition of these KATO III cells by the rhapontin is caused by induction of apoptosis.

Rhapontin has stilbene moiety in the molecule. Apoptosis of prostate cancer cell line by resveratrol having stilbene moiety in the molecule was reported by Lin *et al* (12). To confirm that stilbene moiety in the molecule is essential for

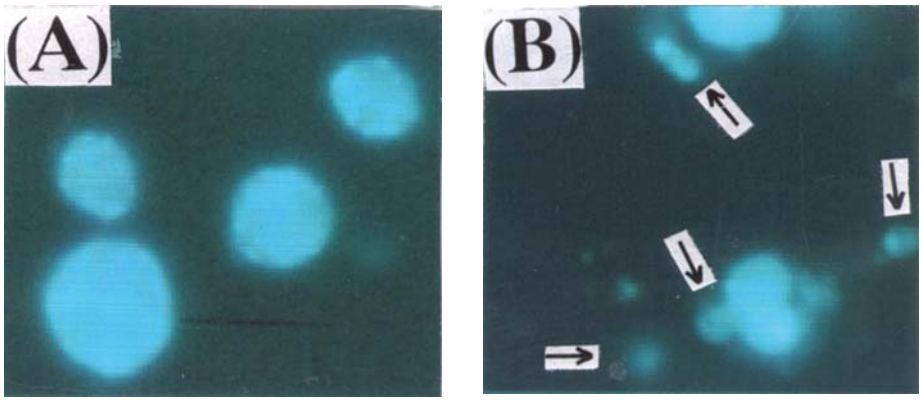

Figure 2. Morphological changes of KATO III cells. (A), Non-treated KATO III cells. (B), KATO III cells treated with 200 μM rhapontin for 3 days. Arrows indicate apoptotic cells.

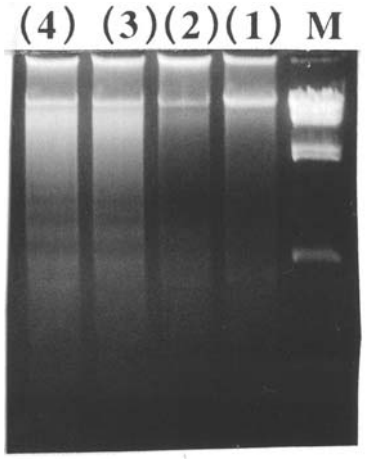

Figure 3. Dose-dependence of DNA fragmentation by rhapontin in the KATO III cells. Cells were cultivated in the presence of a vehicle (lane 1), 50 μM (lane 2), 100 μM (lane 3) and 200 μM (lane 4) rhapontin for 3 days, then washed and harvested. Isolation of DNA from the cells and electrophoresis on 2% agarose gel were carried out as described in Materials and methods. M, λDNA digested with *Hin*dIII.

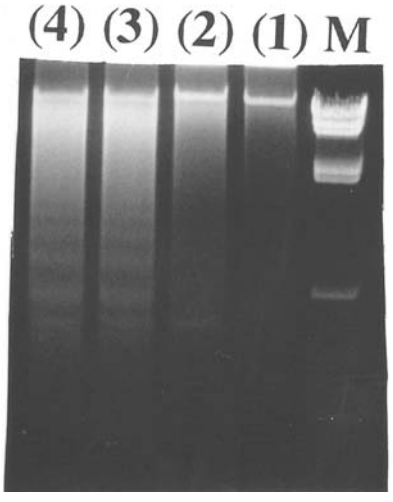

Figure 4. Time-course of DNA fragmentation by rhapontin in the KATO III cells. Cells were cultivated in the presence of a vehicle (lane 1), or 200 μM rhapontin (lanes 2-4) for 1 day (lane 2), 2 days (lane 3) and 3 days (lane 4), then washed and harvested. Isolation of DNA from the cells and electrophoresis on 2% agarose gel were carried out as described in Materials and methods. M, λDNA digested with *Hin*dIII.

the induction of apoptosis, we investigated whether apoptosis by resveratrol was induced or not in the KATO III cells. As shown in Fig. 5, resveratrol also induced apoptosis in the KATO III cells. It is considered that stilbene moiety in the molecule is essential for induction of apoptosis in the KATO III cells.

Mechanism of apoptosis induced by rhapontin and resveratrol. The mechanism of apoptosis induction by rhapontin and resveratrol was investigated. Active oxidants are reported to be a factor responsible for altering the characteristics of mitochondria membrane (13). Shimura *et al* (14) reported on the emittance of apoptosis-inducing factors. They showed that active oxidant was an important factor in apoptosis induction through the non-dependent caspase cascade. In this study it was believed that the active oxidants would work as an apoptosis-inducing signal transfer substance. The active oxidants were attacked by the addition of N-acetyl-L-cysteine, an antioxidant, for 3 days. It was found that N-acetyl-L-cysteine suppressed the DNA fragmentations induced by rhapontin (Fig. 6) and

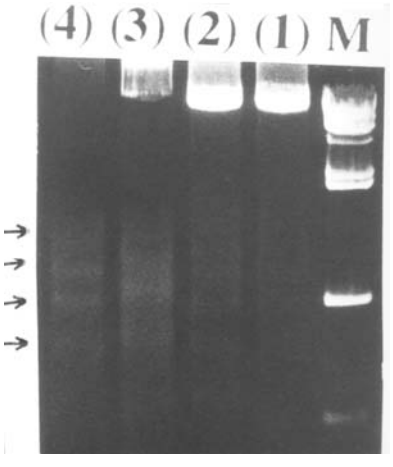

Figure 5. Dose-dependence of DNA fragmentation by resveratrol in the KATO III cells. Cells were cultivated in the presence of a vehicle (lane 1), 50 μM (lane 2), 100 μM (lane 3) and 200 μM (lane 4) resveratrol for 3 days. Isolation of DNA from the cells and electrophoresis on 2% agarose gel were carried out as described in Materials and methods. M, λDNA digested with *Hin*dIII.

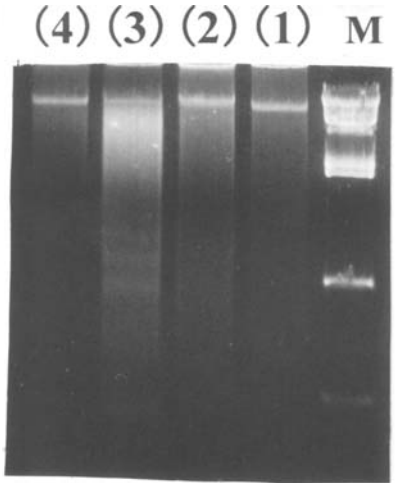

Figure 6. Effect of N-acetyl-L-cysteine, an antioxidant drug, on DNA fragmentation caused by the addition of 200 μM rhapontin to the KATO III cells pre-cultivated with 5 mM N-acetyl-L-cysteine for 2 h. The cells were cultivated in the presence of a vehicle (lane 1), 5 mM N-acetyl-L-cysteine (lanes 2 and 4) and 200 μM rhapontin (lanes 3 and 4) for 3 days. Isolation of DNA from the cells and electrophoresis on 2% agarose gel were carried out as described in Materials and methods. M, λDNA digested with *Hin*dIII.

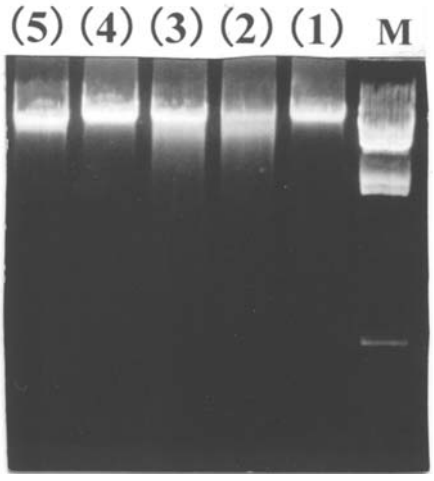

Figure 8. Effects of rhapontin on normal lymphocyte cells. The cells were cultivated in the presence of a vehicle (lane 1), 50 μM (lane 2), 100 μM (lane 3), 200 μM (lane 4) and 300 μM (lane 5) rhapontin for 3 days. Isolation of DNA from the cells and electrophoresis on 2% agarose gel were carried out as described in Materials and methods. M, λDNA digested with *Hin*dIII.

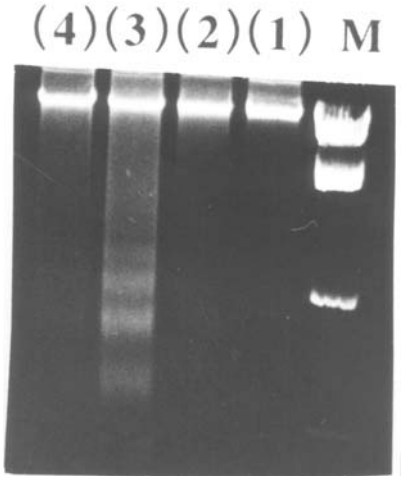

Figure 7. Effect of N-acetyl-L-cysteine, an antioxidant drug, on DNA fragmentation caused by the addition of 200 μM resveratrol to the KATO III cells pre-cultivated with 5 mM N-acetyl-L-cysteine for 2 h. The cells were cultivated in the presence of a vehicle (lane 1), 5 mM N-acetyl-L-cysteine (lanes 2 and 4) and 200 μM resveratrol (lanes 3 and 4) for 3 days. Isolation of DNA from the cells and electrophoresis on 2% agarose gel were carried out as described in Materials and methods. M, λDNA digested with *Hin*dIII.

resveratrol (Fig. 7). These findings suggest that growth inhibition of KATO III cells caused by rhapontin or resveratrol result from the induction of apoptosis caused by these compounds as well as the involvement of active oxygen in these inductions.

In contrast, we have observed no induction of apoptosis by rhapontin in normal lymphocytes prepared from healthy volunteers (Fig. 8).

In general, during apoptosis several characteristic morphological changes are induced, both in the cytoskeleton (leading to bleb formation) and in the nucleus (chromatin condensation and nuclear fragmentation). It has been shown that upon apoptosis, stimulus substance decreases and mitochondria factors (cytochrome C and apoptosis-inducing factor) are released from the mitochondria (15,16). Also a characteristic of apoptosis is the involvement of phosphatidyl serines on the extracellular side of the plasma membrane and the activation of caspase.

The search for better cancer chemotherapeutic agents as well as food that help to protect against stomach cancer is still ongoing all over the world. However, at present, there are no anticancer drugs which are free of side effects in humans. In our experiment, oligonucleosomal-sized DNA fragmentation resulted from the continuous exposure of KATO III cells to the rhapontin. Rhapontin was tested by analyzing its capability of inhibiting the stomach cancer cell proliferation and induction of apoptosis.

In conclusion, these findings demonstrate that rhapontin might exert antitumour activity by triggering apoptosis in the stomach cancer cells. A strategy to selectively induce apoptosis of stomach cancer cells without altering healthy cells in a major goal in the development of new therapeutic techniques.

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