Induction of apoptosis by rhapontin having stilbene moiety, a component of rhubarb (*Rheum officinale Baillon*) in human colon cancer COLO201 cells

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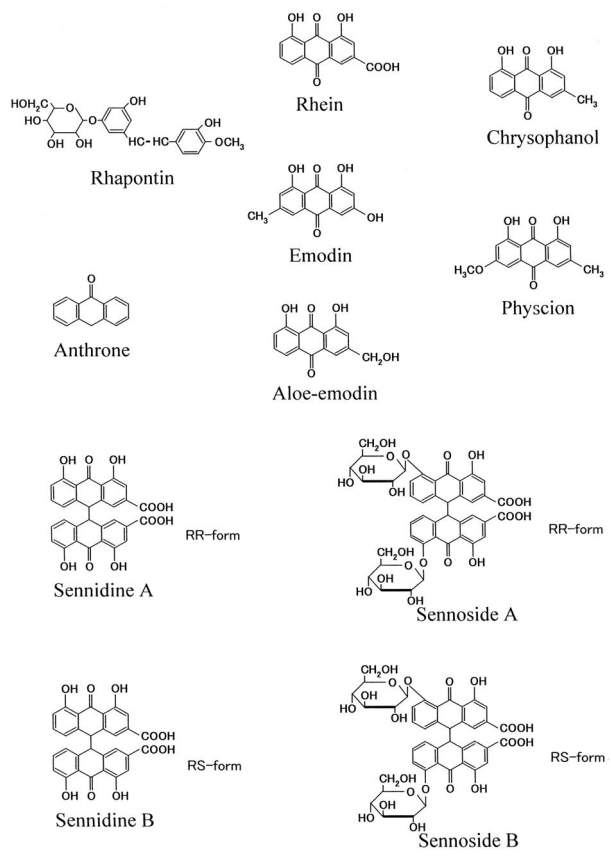
Abstract

We have investigated the effects of rhapontin on proliferation and DNA of human colon cancer COLO201 cells. Growth inhibition and induction of apoptosis by rhapontin were observed in the COLO201 cells. Morphological change showing apoptotic bodies was observed in the COLO 201cells 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.

The data of the present study show that the suppression of COLO 201 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 COLO201 cells.



Various components of rhubard (*Rheum officinale* Baillon)

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 isolate from rhubarb (1).

Thus far we have purified several compounds extracted from plant and evaluated the anti-tumour activity of those compounds (2~7). The efficiency of the anti-tumour compounds seems to be related to the propensity of tumour cells to respond to these compounds by apoptosis.

Recently, considerable attention has been focussed 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 papers 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 paper, we demonstrate for the first time that rhapontin, a component of rhubarb (*Rheum officinale Baillon*), induce apoptosis resulting in suppression of proliferation of human colon cancer COLO201 cells.

Materials and methods

Chemicals.

Rhapontin was 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.

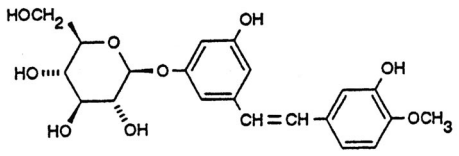


Figure 1. Chemical structure of rhapontin

Cell culture.

Human colon cancer COLO201 cells were originally provided by ATCC and obtained from the Health Science Research Resources Bank (HSRRS), Osaka, Japan. COLO201 cells were grown in RPMI-1640 medium containing 10% fetal bovine serum, penicillin G (50 IU/ml) and streptomycin (50 (g/ml). These cells were cultivated at 37℃ under humidified 95% air - 5% CO2 atmosphere, and passaged every 4 days. Mycoplasma testing was routinely negative. Exponentially growing human COLO201 cells were placed in triplicate at 4 to 5 × 105 cells/ml in the culture flask and cultivated in the presence of a vehicle (50% ethanol) or rhapontin. After cultivation for 1 to 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% phytohemglutinin-M (Gibco Laboratories, Grand Island, NY, USA). 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 COLO201 cells.

Exponentially growing COLO201 cells were placed at the initial density of 4 to 5 × 105 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 (P x L 1400, Phometrics, Ariz, USA) and Fuji pictography 3000 as described by Okumura et al (10). presence of a vehicle or rhapontin for 3 days.

Assay for DNA fragmentation.

Exponentially growing human COLO201 cells were placed at the initial density of 4 to 5 × 105 cells/ml in the culture flask. After cultivation in the presence of a vehicle (50% ethanol) or rhapontin for 1 to 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 COLO201 cells were plated at 4 to 5 x 105 cells/ml. After pre-incubation with N-acetyl-L-cysteine, a known antioxidant, for 2 h, the cells were added with rhapontin 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 1. Effects of rhapontin on the growth of COLO201 cells

	Concentration (μ M)	Inhibition (%)
Vehicle (50% ethanol)	0	0
rhapontin	50	22.8
	100	62.1
	200	99.8

COLO201 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.

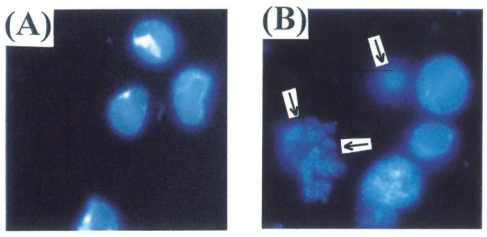


Figure 2.
Morphological changes of COLO201 cells. (A) Non-treated COLO201 cells. (B) COLO201 cells treated with 200 μM rhapontin for 3 days. Arrows indicate apoptotic cells.

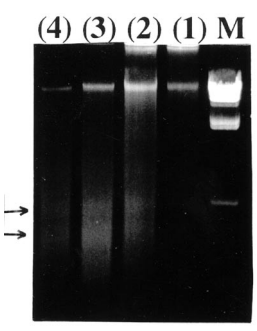


Figure 3.
Dose-dependency of DNA fragmentation by rhapontin in the COLO201 cells. Cells were cultivated in the presence of a vehicle (lane 1), 50 μM (lane 2), 100 μM (lane 3) and 200 μM (lane 4) rhapontin for 3 days, then washed and harvested. Isolation of DNA from the cells and electrophoresis on 2% agarose gel were carried out as described in Materials & Methods. M: λ DNA digested with Hind Ⅲ.

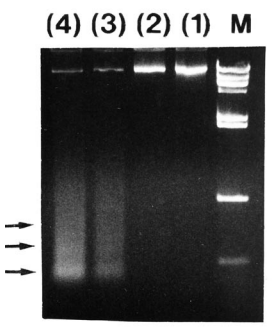


Figure 4.
Time-course of DNA fragmentation by rhapontin in the in the COLO201 cells. Cells were cultivated in the presence of a vehicle (lane 1), or 200 μM rhapontin (lanes 2 ~ 4) for 1 day (lane 2), 2 days (lane 3) and 3 days (lane 4), then washed and harvested. Isolation of DNA from the cells and electrophoresis on 2% agarose gel were carried out as described in Materials & Methods. M: λ DNA digested with Hind Ⅲ.

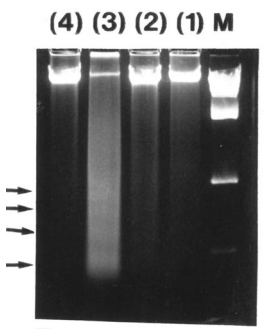


Figure 5.
Effect of N-acetyl-L-cysteine, an antioxidant drug, on DNA fragmentation caused by the addition of 200 μM rhapontin to the COLO201 cells pre-cultivated with 5 mM N-acetyl-L-cysteine for 2 h. The cells were cultivated in the presence of a vehicle (lane 1), 5 mM N-acetyl-L-cysteine (lanes 2 and 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 & Methods. M: λ DNA digested with Hind Ⅲ.

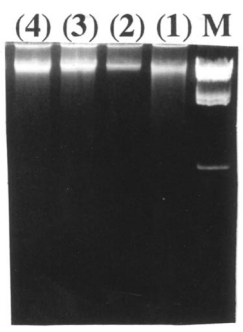


Figure 6.
Effects of rhapontin on normal lymphocyte cells. The cells were cultivated in the presence of a vehicle (lane 1), 50 μM (lane 2), 100 μM (lane 3), 200 μM (lane 4) and 300 μM (lane 5) rhapontin for 3 days. Isolation of DNA from the cells and electrophoresis on 2% agarose gel were carried out as described in Materials and Methods. M: (DNA digested with Hind Ⅲ.

Results and discussion

Effect of rhapontin on the growth of COLO 201 cells.

The effect of rhapontin on the proliferation of human COLO201 cells have been studied. As shown in Table 1, the proliferation of COLO201 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 antitumor 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 COLO201 cells shows apoptotic bodies after 3 days of treatment with 200μM rhapontin (Fig. 2). Rhapontin was specific for the induction of apoptosis in COLO201 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 (data not shown). In the present study, apoptosis by rhapontin in the COLO201 cells was observed for the first time.

Dose-dependency and time-dependency of apoptosis by rhapontin.

As shown in Fig. 3, oligonucleosomal-sized fragments were observed in the COLO201 cells treated with rhapontin. Induction of apoptosis by rhapontin in the COLO201 cells was occurred in dose-dependent (from 50 to 200μM) (Fig. 3) and time-dependent (from 2 to 3 days) (Fig.4) manners. From these findings it is considered that growth inhibition of these COLO201 cells by the rhapontin is caused by inducing apoptosis.

Mechanism of apoptosis induced by rhapontin.

The mechanism of apoptosis induction by rhapontin 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. 5). These findings suggest that growth inhibition of COLO201 cells caused by rhapontin result from the induction of apoptosis caused by this compound as well as the involvement of active oxygen in this induction.

In contrast, we have observed no induction of apoptosis by rhapontin in normal lymphocytes prepared from healthy volunteers (Fig. 6).

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). Recently, it was shown that upon apoptosis, stimulus substance decreases and mitochondria factors (cytochrome C and apoptosis-inducing factor) are released from the mitochondria (15, 16). Also 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 foods 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, oligo-nucleosomal-sized DNA fragmentation resulted from the continuous exposure of COLO201 cells to the rhapontin. Rhapontin was tested by analyzing their capability of inhibiting these colon cancer cell proliferation and of inducing apoptosis.

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

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