Camelliin B induced apoptosis in HeLa cell line

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Abstract

Gordonia axillaris (Roxb.) Dietrich (Theaceae) is a native to Taiwan and the leaves have been used as an astringent folk medicine. Camelliin B (CB), a macrocyclic hydrolyzable tannin, was isolated from G. axillaris and showed cytotoxic effects in human carcinoma cells. Among the target cells (SKHep-1, Ha-22T, DU-145, AGS, and HeLa), the cervical carcinoma cell line, HeLa, was more sensitive to CB than were Chang normal liver cells and primary-cultured normal gingival and cervical fibroblasts. Furthermore, the cytotoxic effects of CB showed dose-dependency at 3.2–100.0 µg/ml in HeLa for 1, 24, 48, and 72 h and with an IC50 value of 46.3 µg/ml for 48 h. However, the IC50 value of CB in primary-cultured normal cervical fibroblasts was 108.0 µg/ml. Therefore, the selectivity shown by CB was ascribed to the difference in growth speed between normal and tumor cells. HeLa cells and primary-cultured normal cervical fibroblasts were treated with 50.0 and 100.0 µg/ml CB for 48 h, respectively, and exhibited chromatin condensation, indicating the occurrence of apoptosis. Flow cytometric analysis demonstrated the presence of apoptotic cells with low DNA content, a decrease of cell population at the G1 phase, and a concomitant increase of cell population at the G2/M phase. CB also caused DNA fragmentation and inhibited PARP degradation in HeLa cells. However, CB did not significantly inhibit Bcl-2 expression in HeLa cells at 50.0 µg/ml, only at 100.0 µg/ml for 48 h. These results suggest that CB induced apoptosis, without direct inhibition of Bcl-2 expression in HeLa cells. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Natural products have been used in traditional and folk medicine for therapeutic purposes. Several anticancer drugs derived from natural sources, including the well-known Cantharanthus alkaloids, colchicine, etoposide, and taxol (Pezzuto, 1997), have been used in cancer chemotherapy. Since cytotoxic and anti-proliferative drugs have had great success and will likely continue to play a major role in cancer treatment, we are interested in finding new active compounds from natural sources and studying their biological effects.

G. axillaris (Roxb.) Dietrich (Theaceae) is a small tree native to Taiwan. The leaves of G.
axillaris have been used for stomachache, diarrhea, arthritis and as an astringent folk medicine in Taiwan and South Africa (Power et al., 1991; Chang et al., 1994). In a previous report, we isolated and characterized a macrocyclic hydrolyzable tannin dimer, camelliin B (CB), from the leaves of this plant (Chang et al., 1994). Hydrolyzable tannins have been shown to have antiviral (Nakashima et al., 1992), antimicrobial (Burapadaja and Bunchoo, 1995), antioxidant (Sato and Sakagami, 1996), and hepatoprotective activities (Miyamoto et al., 1993a) as well as antitumor effects (Haslam, 1996). The potency of the activities depends on the structure of the tannins. Tannins with macrocyclic structures were reported to have antitumor activity and significant host-mediated antitumor activity which suggests that macrocyclic tannins may exert their antitumor effect by enhancing the immune response of the host (Miyamoto et al., 1988, 1993b,c; Yoshida et al., 1989; Sakagami et al., 1990, 1992; Murayama et al., 1992; Kashiwada et al., 1992; Feldman et al., 1999; Wang et al., 1999). However, the underlying mechanism of the antitumor activity induced by CB has not yet been clarified.

The purpose of this study was to determine the extent to which the macrocyclic ellagitannin, CB, inhibits the growth of human carcinoma cell lines in culture, and we attempted to determine whether CB would induce tumor cell death in vitro.

It is clearly of interest to examine whether the cytotoxic activity of CB is a general phenomenon in human cells, with activity against various kinds of malignant cells and normal cells. Moreover, its potential as an agent capable of causing apoptotic cell death warrants further examination. Therefore, the present assessment of the cytotoxic activity of CB in human cancer cell lines (SKHep-1, Ha-22T, DU-145, AGS, and HeLa), Chang normal liver cell line and primary-cultured normal cervical and gingival fibroblasts was performed. The apoptosis-inducing activity was also tested.

Apoptosis, or programmed cell death, is a highly regulated process that involves the activation of a cascade of molecular events leading to cell death that is characterized by plasma membrane blebbing, shrinkage, chromatin condensation, chromosomal DNA fragmentation, and formation of membrane-bound apoptotic bodies that are eventually phagocytosed by neighboring cells (Allen et al., 1997). Accordingly, we observed the morphology of the tumor cells in response to treatment by light microscopy, and then measured the DNA fragmentation by biochemical analyses.

2. Materials and methods

2.1. Materials

Camelliin B was isolated from G. axillaries (Roxb.) Dietrich (Theaceae) and was analytically pure as shown by high-performance liquid chromatography and nuclear magnetic resonance spectra (Chang et al., 1994). Dimethyl sulfoxide (DMSO), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), trypan blue, and other chemicals were purchased from Sigma Industries (St. Louis, MO, USA). Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotics, glutamine, and trypsin-EDTA were purchased from Gibco (Grand Island, NY, USA). Western blot was performed by using antibody specific to human Bcl-2 (sc-783), poly(ADP-ribose)polymerase (PARP, sc-7150), β-tubulin (sc-8035), anti-rabbit IgG-AP (sc-2007) and anti-mouse IgG-AP (sc-2008) which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents and chemicals were of the highest purity grade available (Fig. 1).

2.2. Cell cultures

The human hepatocellular carcinoma, SKHep-1 and Ha-22T; human prostate carcinoma, DU-145; human gastric adenocarcinoma, AGS and human cervical carcinoma, HeLa, cell lines were obtained from American Type Cell Culture (ATCC) (Rockville, MD, USA). Human tumor cell lines were maintained in DMEM (Gibco) supplemented with 10% FBS, 100.0 mg/l streptomycin, and 100 IU/ml penicillin (Gibco). The cell cultures
were incubated at 37 °C in a humidified atmosphere of 5% CO₂.

2.3. Primary culture

Normal fibroblasts were isolated from human normal gingival and cervical tissues, respectively. The fresh tissues were rinsed twice with phosphate-buffered saline (PBS), and then cut to 0.1 cm³ pieces. These pieces were incubated with 0.25% trypsin and 1.5 mg/ml type II collagenase in DMEM for 4 h at 37 °C with 5% CO₂. Cells were then harvested in DMEM containing 10% FBS, 100 mg/l streptomycin, and 100 IU/ml penicillin centrifuged for 10 min at 1200 rpm, and then seeded in culture dishes. After 1 day, the medium was changed to eliminate floating cells. Cells were used for experimental protocols from passage 1 to 3.

2.4. Cytotoxicity assays

The stock solution of CB (2 × 10⁴ µg/ml) was prepared by dissolving CB in dimethyl sulfoxide (DMSO) and then storing it at 4 °C until use. Serial dilutions of the stock solution were prepared in the culture medium in 96-well microtiter plates. CB at the appropriate concentrations was added to cell cultures (1 × 10⁵ cells/well) for 36 h without renewal of the medium. The number of surviving cells was then counted by using the tetrazolium (MTT) assay (Rubinstein et al., 1990). Finally, the products were evaluated by measuring the optical density for each well at 600 nm, using an MRX microplate reader (Dynex Technologies, Guernsey, Channel Islands, Great Britain, UK). Cytotoxicity Index (CI%) was calculated according to the following equation:

\[ \text{CI\%} = \left[ 1 - \left( \frac{T}{C} \right) \right] \times 100\% \]

where \( T \) and \( C \) represent the mean optical density of the treated group and vehicle control group, respectively. In accordance with the CI% of the dose–response curve the concentration of the test compound giving 50% of cell growth inhibition (IC₅₀ value) was estimated.

2.5. Morphological analysis

CB-treated cells were washed twice with PBS and fixed in methanol for 10 min. The fixed cells were spread on a glass slide and stained with Giemsa-White staining solution. Morphological changes were detected by light microscopy.

2.6. Flow cytometry analysis

After appropriate treatment, HeLa cells (5 × 10⁵ cells/well) were harvested by centrifugation and washed with PBS. The cells were fixed with ice-cold 80% ethanol for 30 min, washed with PBS, and then treated with 0.25 ml of 0.5% Triton X-100 solution containing 1.0 mg/ml RNase A at 37 °C for 30 min. Finally, 0.25 ml of 50 µg/ml propidium iodide was added to the sample for 30 min in the dark (Allen et al., 1997). Samples were run through a FACScan (Becton Dickinson, San Jose, CA, USA). Results are presented as the number of cells versus the amount of DNA as indicated by the intensity of fluorescence.

Fig. 1. Structure of camellin B (M.W. 1713) isolated from G. axillaries (Roxb.) Dietrich.
2.7. Agarose gel electrophoresis

HeLa cells ($5 \times 10^5$ cells/well) exposed to CB for 48 h were collected into tubes and then washed with PBS. The cells were incubated for 10 min in 200 μl lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% Sarkosyl) at room temperature, then centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was incubated overnight at 56 °C with 250 μg/ml proteinase K. Cell lysates were then treated with 2 mg/ml RNase A and incubated at 56 °C for 1.5 h. DNA was extracted with 1 volume of chloroform/phenol/isoamyl alcohol (25:24:1), and precipitated from the aqueous phase by centrifugation at 14,000 × g for 30 min at 0 °C. An aliquot (10–20 μl) of this solution was transferred to a 1.8% agarose gel containing 0.5 μg/ml of ethidium bromide, and electrophoresis was carried out at 80 V for 2 h with TBE (×0.5) as the running buffer. DNA in the gel was visualized under UV light (Allen et al., 1997).

2.8. Western blot analysis

HeLa cells ($5 \times 10^5$ cells/well) exposed to CB for 48 h were collected into tubes and then washed with PBS. Cell pellets were lysed with lysis buffer containing 40 mM Tris–HCl (pH 7.4), 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% Nonide P-40, and protease inhibitors. Total proteins (50 μg) were used for Western blot analysis. Western blot analysis was performed using 10% Tris-glycine-SDS-polyacrylamide gels, and the protein was transferred to a nitrocellulose membrane by electroblotting. The membranes were probed with anti-Bcl-2 (a rabbit polyclonal antibody) and anti-PARP (a rabbit polyclonal antibody), and visualized using a BCIP/NBT kit (BCIP/NBT, Gibco) according to the manufacturer’s instructions. As a loading control, we used anti-α-tubulin (a mouse monoclonal antibody).

3. Results

3.1. Cytotoxic effect of camelliin B

When the five human carcinoma cell lines (SKHep-1, Ha-22T, AGS, DU-145, and HeLa), Chang normal liver cell line, and primary culture gingival and cervical fibroblasts were treated with CB at 100.0 μg/ml for 36 h at 37 °C, cytotoxic activity was observed by MTT assay. Camelliin B showed different cytotoxic effects on the various carcinoma cell lines with the greatest sensitivity shown by HeLa cells in Fig. 2. Two of the tumor cell lines are resistant (SKHep-1 and Ha-22T), whereas three are sensitive (DU-145, AGS, and HeLa). Among the three types of normal cells one is resistant (PGF), and two are sensitive (CNL and PCF). Moreover, CB was less cytotoxic on primary culture gingival fibroblasts than on cancer cell lines at 100.0 μg/ml (Fig. 2). Fig. 3 shows CB-inhibited HeLa cell proliferation in a concentration- and time-dependent manner at 3.2–100.0 μg/ml on HeLa for 1, 24, 48, and 72 h. If HeLa cells were exposed to CB for short interval (<24 h), the IC₅₀ was more than 100.0 μg/ml. However, when HeLa cells were treated with CB for more than 48 h, the IC₅₀ was exhibited below 50.0 μg/ml (IC₅₀ = 46.3 μg/ml for 48 h). Nevertheless, the IC₅₀ value of CB in primary-
cultured normal cervical fibroblasts was 108.0 μg/ml for 48 h (Fig. 4). The selectivity shown by CB was ascribed to the difference in a dosage between normal and tumor cells. Lethal dosage of CB in primary-cultured normal cervical fibroblasts was two folds higher than in HeLa cells.

3.2. Effect of camelliin B on morphology

To characterize the cell death of HeLa and primary culture normal cervical fibroblasts, we further examined morphological changes in the cells in detail using a light microscope. Fig. 5 shows the nuclear condensation in HeLa cells exposed to 50.0 μg/ml CB for 48 h by Giemsa stain. When primary culture normal cervical fibroblasts were treated with 100.0 μg/ml for 48 h, nuclear condensation shown was seen in a few cells, but a lot of cells were intact. Condensation of the chromatin is known to reflect sequential stages of apoptosis. According to Fig. 5, HeLa cells were more sensitive to CB induced apoptosis than primary culture normal cervical fibroblasts.

3.3. Effect of camelliin B on DNA fragmentation

HeLa cells were treated with a range of CB concentrations, and the cell cycle distribution was examined after 24 or 48 h. When the dosage was 100.0 μg/ml, the DNA content-frequency histograms showed a sub-G1 peak and a progressive loss of the normal G1 phase (Fig. 6). However, the low concentration (25.0 μg/ml) had to be cultured for 48 h before HeLa cells showed a sub-G1 peak (Fig. 6). CB induced apoptosis in HeLa cells was also confirmed by flow cytometric analysis of DNA-stained cells (Fig. 6).

DNA fragmentation is a characteristic feature of apoptosis (Allen et al., 1997). Increased DNA fragmentation was apparent in HeLa cells after treatment with 12.5–100.0 μg/ml CB for 48 h. A typical experimental result of agarose gel electrophoresis is shown in Fig. 7, where the effect of 50.0 and 100.0 μg/ml CB for 48 h treatment produced DNA fragment ladders.

3.4. Effect of camelliin B on cleavage of poly(ADP-ribose)polymerase (PARP)

Since the proteolytic cleavage of PARP is known to be a hallmark of apoptosis, degradation of PARP during taxol treatment was investigated by Western blot for some cell lines (Yeung et al., 2000). As shown in Fig. 8, the 115 kDa protein was cleaved to give a typical 85 kDa fragment when the HeLa cell line was incubated with 12.5–100.0 μg/ml CB for 48 h. On the other hand, the results showed that HeLa cells exposed to 100.0 μg/ml CB for 48 h (Fig. 8) contained significantly lower levels of Bcl-2 protein compared to HeLa cells treated with the same concentration of DMSO (0.3%). These results suggested that CB
Fig. 5. Morphological alterations in HeLa cells and PCF after CB exposure: (A) control HeLa cells (200 ×); (B) HeLa cells were treated with 50.0 μg/ml for 48 h (200 ×); (C) control PCF (200 ×); (D) PCF were treated with 100.0 μg/ml for 48 h (200 ×) PCF, primary normal cervical fibroblasts. Black arrows indicate chromatin condensation. The data was detected from three separated experiments and showed one picture of them.
induced apoptosis, without direct inhibition of Bel-2 expression in HeLa cells.

4. Discussion

Camelliin B was initially isolated from *Camellia japonica* and exhibits marked host-mediated antitumor effects (Yoshida et al., 1989). In a previous study, we also isolated CB from *G. axillaries* (Chang et al., 1994) and investigated the effects of CB on proliferation, viability, and cell cycle progression of representative CB-sensitive tumor cell lines.

In an in vitro assay, CB was more cytotoxic to HeLa than to normal cervical fibroblasts. The mechanism of CB induced HeLa cell death was explored in this study. According to the results of flow cytometry analysis and agarose gel electrophoresis, CB showed degenerative DNA phenomena (Figs. 6 and 7). Moreover, the morphology of CB-treated HeLa cells showed chromatin condensation as observed by light microscopy (Fig. 5). Furthermore, CB also induced apoptosis in normal cervical fibroblasts at 100.0 μg/ml for 48 h as shown in Fig. 5. Apoptosis produced the typical pattern of apoptotic PARP cleavage: a catalytically active band of intact PARP at 116 kDa, and an active band at 85 kDa corresponding to the apoptotic cleavage product of PARP. PARP is proteolytically

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Fig. 6. DNA content-frequency histograms of HeLa cells after treatment with 25, 50 and 100.0 μg/ml CB for 24 and 48 h. The data was detected from three separated experiments and showed one picture of them.
cleaved during apoptosis by caspase-3 (Tewari et al., 1995) which reduces PARP's enzymatic activity (Lazebnik et al., 1994), thereby inhibiting DNA repair. In Fig. 8, CB-treated HeLa cells show a dose-dependent increase in the cleavage of PARP (85 kDa) which might indicate a breakdown in the DNA repair function. Moreover, Bcl-2 and its homologs are critical regulators of the cell death pathway (Hockenbery et al., 1990). HeLa cells were treated with CB only at a high concentration (100.0 μg/ml) that showed the inhibition of Bcl-2 expression. These results suggested that CB induced apoptosis, without direct inhibition of Bcl-2 expression in HeLa cells.

Many compounds have been shown to be capable of inhibiting proliferation of mammalian cells in culture. However, only a small proportion of cytotoxic compounds demonstrate significant selectivity in vivo even in the most chemosensitive animal tumor models. Previous studies showed that tannin could induce interleukin-1 (IL-1) production from human peripheral macrophages in vitro (Murayama et al., 1992; Sakagami et al., 1992; Miyamoto et al., 1993b, c; Kashiwada et al., 1992; Feldman et al., 1999). When tannin (10 mg/kg) was intraperitoneally injected into mice once, 4 days before intraperitoneal inoculation with S-180 cells, macrocycle ellagitannins (einothein B, woodfordin C, camelliin B, cuphiins D1, and D2) had significant antitumor effects (Miyamoto et al., 1993a; Kashiwada et al., 1992; Wang et al., 1999).

The structure of CB is that of a macrocyclic ellagitannin and consists of tellimagrandins I and II. Recently, many biological and pharmacological activities of ellagitannins have been reported. Miyamoto reported that most of the oligomeric ellagitannins, consisting of tellimagrandins I and II as the monomer unit, had significant antitumor activity. Macrocyclic ellagitannins were all active. For example, Hirtellin C, woodfordin C, einothein B, cuphiins D1 and D2, and other macrocyclic hydrolyzable tannins have antitumor effects in vivo and in vitro (Miyamoto et al., 1993a; Kashiwada et al., 1992; Wang et al., 1999). Haslam also suggested that the molecular size of the polyphenol is important; in the galloyl-D-glucose series, the efficacy of binding increases as the number of galloyl groups increases in the order of: tri < tetra < penta (Haslam, 1996). Moreover, as to hydrolysable tannin-induced DNA fragmentation, the highest activity was detected in gallic acid, a component unit of tannin (Sakagami et al., 1995).

On the other hand, woodfordin C was reported to be a novel inhibitor of topoisomerase II (Kuramochi-Motegi et al., 1992), and einothein B was found to be a potent and specific inhibitor of poly(ADP-ribose)glycohydrolase (Aoki et al., 1995). In previous studies, cuphiin D1, penta-galloyl-D-glucose, and tannin acid induced apoptosis in HL-60 cells (Sakagami et al., 1995; Wang et al., 2000; Inoue et al., 1994; Pan et al., 1999). Therefore, there was a possibility that CB would be found to induce apoptosis in HeLa cells and to prolong the life of S-180-bearing mice (Miyamoto et al., 1993a) because of its distinctive structures, but the detailed
mechanism is currently unclear. In the future, we will perform structure-activity analysis and explore the mechanisms of its antitumor effects.

The role of apoptosis in the development of malignant cells and its application to cancer therapy has recently been discussed (Thompson, 1995). CB could induce apoptosis in HeLa cells in vitro, prolongs the life of S-180 bearing mice (Miyamoto et al., 1993a) and enhances IL-1β secretion in mice (Miyamoto et al., 1993c). The cervical carcinoma cell line HeLa is more sensitive than primary cervical fibroblast. From our in vitro studies a partial selectivity of CB towards cervical carcinoma cells (HeLa) compared to normal cervical fibroblasts was observed. Extended in vivo studies are warranted to show whether the compound is a candidate to be developed as an anticancer drug.

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References


