Mechanism of Growth Inhibitory Effect of Cape Aloe Extract in Ehrlich Ascites Tumor Cells

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Summary Cape aloe (*Aloe ferox* Miller) has been a herb well known for its cathartic properties and has also been used popularly as a health drink (juice, tea and tonic) in the United States and in Europe. Cape aloe extract also has been reported to possess several pharmacological effects, such as anti-inflammatory, anti-bacterial, anti-fungal and protective effect against liver injury. However, the investigations on an anti-tumor activity in cape aloe extract are very few and subsequent mechanisms have not been well elucidated. In this study, we examined the effect of the selective growth inhibitory activity of cape aloe extract and found that the cape aloe extract, especially the dichloromethane (CH₂Cl₂) extract, caused a dose-dependent growth inhibitory effect in Ehrlich ascites tumor cells (EATC), but not in mouse embryo fibroblast (NIH3T3) cells, which was used as a normal cell model. Furthermore, the CH₂Cl₂ extract caused an accumulation of cells in the G1 phase and a decrease of cells in the S and G2/M phase of the cell cycle and inhibited DNA synthesis in a dose-dependent manner. In addition, other results suggest that cell cycle arrest and inhibition of proliferation in EATC by the CH₂Cl₂ extract are associated with decreased retinoblastoma protein (Rb) phosphorylation.

Key Words cape aloe (*Aloe ferox* Miller), cell cycle, retinoblastoma protein, Ehrlich ascites tumor cells

Cancer chemoprevention is one of the promising methods for cancer control. Studies and interests in cancer chemoprevention by the biological activity and pharmaceutical value of naturally occurring substances, which were derived from food and medicinal plants, have increased in recent decades. In particular, the discovery of natural products with specific action on tumor cells would be helpful in cancer chemoprevention or chemotherapy. Demonstrating the benefits of food by scientific means remains a challenge, particularly when compared with standards applied for assessing pharmaceutical agents. Pharmaceutical agents are small-molecular-weight compounds consumed in a purified and concentrated form. Food is eaten in combinations, in relatively large, unmeasured quantities under highly socialized conditions. However, food-derived products have high potential for development as chemopreventive and therapeutic agents that may find widespread and long-term use (1–4). We previously reported that a green tea extract (5) and evening primrose extract (6) have effective anticancer properties. Furthermore, we screened 31 edible plants, especially

spice and herb extracts, to examine their growth inhibitory activity against Ehrlich ascites tumor cells (EATC) and showed that aloe extract possesses a strong growth inhibitory activity (7).

Aloe has long been used as a medicinal plant and as a health food, and has maintained its popularity over the course of time. In Japan, aloe vera or kidachi aloe has generally been widely utilized for several diseases as a folklore medicine and as an over-the-counter drug. Aloe extracts have also been used as health and nutrition food (8). Similarly, cape aloe (*Aloe ferox* Miller), which is extensively cultivated in South Africa, has been a herb well known for its cathartic properties and has also been used popularly as a health drink (juice, tea and tonic) in the United States and in Europe. The extracted and spray-dried leaf gel is used in skin care products (9–12). In addition, cape aloe has several pharmacological effects, such as anti-inflammatory (13), anti-bacterial and anti-fungal (14) and protective effect against liver injury (15). However, the precise mechanism of these pharmacological effects, especially the anti-tumor activity, and the effective component in cape aloe, are not yet known.

In the present study, we examined the effect of the selective growth inhibitory activity of cape aloe extract

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and its related mechanisms of cell growth arrest in EATC.

MATERIALS AND METHODS

Materials. The commercial cape aloe powder, the dried exudates from *Aloe ferox* Miller, was purchased from Mikuni Co., Ltd. (Osaka, Japan). Monoclonal mouse anti-Rb antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal mouse anti-bromodeoxyuridine (BrdU) antibody, biotinylated goat anti-mouse immunoglobulins and horseradish peroxidase-conjugated streptavidin were purchased from DAKO A/S (Glostrup, Denmark). Fetal bovine serum (FBS) was obtained from JRH Bioscience Inc. (Kansas, USA). Eagle's minimum essential medium and Dulbecco's modified Eagle's medium were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Other chemicals were of the highest analytical grade and purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Extraction and fractionation. Cape aloe powder (200 g) was successively extracted with *n*-hexane (HX, 6×800 mL) and dichloromethane (CH₂Cl₂, 6×800 mL) and 70% aqueous acetone (1×800 mL) at room temperature. For each extraction, the sample material was soaked in the solvent and allowed to stand overnight. The HX soluble part and CH₂Cl₂ soluble part were evaporated in vacuo to give the HX extract (0.12 g) and CH₂Cl₂ extract (0.67 g). The aqueous acetone layer was concentrated in vacuo, and the resulting aqueous solution was partitioned with ethyl acetate (EtOAc) to give the EtOAc soluble fraction (41.6 g), water (H₂O) soluble fraction (77.2 g) and the EtOAc and H₂O insoluble fraction (EtOAc/H₂O insoluble, 57.6 g). Each of the fractions was evaporated to dryness in vacuo (Fig. 1).

Cell culture. EATC were cultured in a humidified atmosphere of 5% CO₂ in air at 37°C for 3–4 d in Eagle's minimum essential medium containing 10% FBS. The cells were washed and cultured again at a concentra-

tion of 1×10⁶ cells/mL in fresh medium. Mouse embryo fibroblast (NIH3T3) cells were cultured in a humidified atmosphere of 5% CO₂ in air at 37°C for 3 d in Dulbecco's modified Eagle's medium containing 10% FBS. Then the cells were washed and cultured again at a concentration of 1×10⁵ cells/mL in fresh medium. Each extract of the cape aloe powder was dissolved in dimethyl sulfoxide (DMSO) and diluted in the culture medium immediately before use (final DMSO concentration <0.5%). In all the experiments control cultures were made up of medium, DMSO and the cells only. DMSO alone did not have any effect on the parameters measured.

Assay of cell viability and cell proliferation. Cell viability of EATC was determined by the trypan blue exclusion assay as described (16). The trypan blue exclusion assay is the most commonly utilized test of cell viability and cell proliferation. In particular, this method is more effective for the measurement of both viable and non-viable (dead) cell in the EATC suspension. Briefly, EATC (1×10⁶ cells/mL) treated with cape aloe extract at various concentrations were incubated in a humidified atmosphere of 5% CO₂ in air at 37°C for 24 h in Eagle's minimum essential medium containing 10% FBS. An equal volume of 0.4% trypan blue reagent was added to the cell suspension and the percentage of viable cells was evaluated under a field microscope. Similarly, assay of cell proliferation of EATC was determined by the trypan blue exclusion method. Cell proliferation was indicated by the total cell number. Cell viability of NIH3T3 cells, used as a representative normal cell line, was measured by the neutral red (NR) assay described previously (17). The NR assay is capable of measuring cell death for NIH3T3 cells, which are attached to cell culture dishes. NIH3T3 cells (1×10⁵ cells/mL) treated with the CH₂Cl₂ extract of cape aloe at various concentrations were incubated in a humidified atmosphere of 5% CO₂ in air at 37°C for 24 h in Dulbecco's modified Eagle's medium containing 10% FBS. NR stock solution (0.4% NR in water) was diluted 1:80 in phosphate-buffered saline (PBS). NIH3T3 cells were incubated with the NR solution for 2 h at 37°C to allow the lysosomes of viable cells to take up the dye. The NR solution was then removed and the cultures were washed rapidly with a mixture of 1% formaldehyde–1% calcium chloride. A mixture of 1% acetic acid–50% ethanol was added to extract the NR from the cells at room temperature for 30 min. Each sample was then measured at 540 nm with a spectrophotometer (JASCO V-530). All analyses were performed in triplicate.

Measurement of DNA synthesis. DNA synthesis was visualized by the incorporation of BrdU and the immunohistochemical detection of the nucleotide analogue. EATC were treated with the CH₂Cl₂ extract of cape aloe at various concentrations for 5 h and then were incubated with 50 μM BrdU for 19 h at 37°C. The cells were fixed with 5% acetic acid in 95% ethanol, overnight at 4°C. The fixed cells were re-incubated with formamide at 70°C for 45 min and then were smeared on slide glass. The specimens were incubated with the primary

Fig. 1. Extraction and fractionation of cape aloe.

antibody, monoclonal anti-BrdU antibody (1:50 dilution) for 1 h at room temperature and then with biotinylated anti-mouse goat immunoglobulins for 1 h followed by incubation with horseradish peroxidase-conjugated streptavidin for 1 h. PBS was used for washing between the subsequent incubation steps. For peroxidase reaction, 3,3'-diaminobenzidine tetrahydrochloride with nickel chloride color modification was incubated for 5 min until the desired color intensity had developed. The number of BrdU-positive nuclei was counted in three microscopic fields in each specimen.

Measurement of cell cycle analysis. The CH_2Cl_2 extract of cape aloe-induced cell cycle changes were analyzed using a laser scanning cytometer (LSC 101; Olympus Optical Co. Ltd., Tokyo, Japan) using propidium iodide (PI) staining. Briefly, after designated treatments, EATC were fixed with 70% ethanol for 30 min and incubated with freshly prepared PI-stained buffer (0.1% Triton X-100 in PBS, 50 $\mu\text{g}/\text{mL}$ PI, 150 $\mu\text{g}/\text{mL}$ RNase) for 1 h at 37°C in the dark (18).

Preparation of protein for Western blot analysis of Rb. The CH_2Cl_2 extract of cape aloe-treated EATC was washed twice in PBS and resuspended in 100 μL of lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM ethylenediaminetetra-acetic acid (EDTA), 0.5% sodium deoxycholate, 1% Nonidet P-40, 10 $\mu\text{g}/\text{mL}$ pepstatin, 1 mM sodium vanadate, 50 $\mu\text{g}/\text{mL}$ leupeptin, 20 $\mu\text{g}/\text{mL}$ aprotinin, 100 $\mu\text{g}/\text{mL}$ phenylmethylsulfonyl fluoride, pH 7.5) for 20 min on ice followed by freeze-thawing three times. Lysates were centrifuged at $17,500 \times g$ for 20 min at 4°C and the supernatant was collected for Western blot (18). Protein concentrations were determined by the Bradford method (19).

Western blotting analysis of Rb. Equal amounts of protein for Rb assay were loaded into each lane of a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and the separated proteins were blotted to 0.45 μm polyvinylidene fluoride membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). After an overnight blocking with 5% non-fat milk, 0.1% Tween-20, in PBS, the membrane was incubated with the primary antibody, monoclonal anti-Rb antibody (1:500 dilution) for 1 h at room temperature and then with biotinylated anti-mouse goat immunoglobulins (1:750 dilution) followed by incubation with horseradish peroxidase-conjugated streptavidin (1:750 dilution) for 1 h at room temperature. PBS was used for washing between the subsequent incubation steps. Horseradish peroxidase activity was visualized with 3-amino-9-ethylcarbrazol (18). Densitometry analysis of the protein bands was performed with the software Scion Image (Scion Corporation).

Statistical analysis. Data are represented as means $\pm$ SD (standard deviation from the mean). The significance of difference in the assay was evaluated with ANOVA followed by Tukey multiple tests. $p < 0.01$ or $p < 0.05$ was used to indicate a statistically significant difference.

RESULTS

Effect of cape aloe extract on cell viability and cell proliferation of EATC

Cape aloe powder was extracted with HX, CH_2Cl_2 , EtOAc and H_2O and the effect of each extract (200 $\mu\text{g}/\text{mL}$) on cell viability and cell proliferation was examined in EATC by the trypan blue method. The CH_2Cl_2 extract decreased cell viability (Fig. 2A) and total cell number (Fig. 2B) significantly and more than the other extracts and non-treated cells. The effect of various doses of the CH_2Cl_2 extract of cape aloe (0–200 $\mu\text{g}/\text{mL}$) on cell viability and total cell number is shown in Fig. 3A, B. The CH_2Cl_2 extract decreased cell viability and total cell number in a dose-dependent manner as well.

Effect of CH_2Cl_2 extract on cell viability of NIH3T3 cells

NIH3T3 cells were used as a representative normal cell model to analyze selectivity of toxicity. The effect of various doses of the CH_2Cl_2 extract (0–200 $\mu\text{g}/\text{mL}$) on

Fig. 2. Effect of various fractions of cape aloe extract on cell viability and cell proliferation of EATC. EATC were cultured in Eagle's minimum essential medium containing 10% FBS for 3–4 d, were diluted and incubated again in fresh medium with each aloe extract (200 $\mu\text{g}/\text{mL}$) for 24 h. Control cultures contain DMSO (final concentration $<0.5\%$) and medium. Cell viability (A) and total cell number (B) were measured 24 h later by the trypan blue exclusion method. The total cell number means the sum of viable and dead cells. Broken lines in (B) represent the total cell number in 0 h. Results show means $\pm$ SD of three different experiments. The differences between means were significant at ** $p < 0.01$ or * $p < 0.05$, compared with non-aloe extract treated cultures.

Fig. 3. Dose-dependent effect of the CH_2Cl_2 extract on cell viability and cell proliferation of EATC. EATC were cultured in Eagle's minimum essential medium with various concentrations of the CH_2Cl_2 extract of cape aloe and harvested at 24 h. Cell viability (A) and total cell number (B) were as measured with the trypan blue method. The total cell number means the sum of viable and dead cells. Broken lines in (B) represent the total cell number in 0 h. Results show means $\pm$ SD of three different experiments. The differences between means were significant at ** $p < 0.01$, compared with non- CH_2Cl_2 extract treated cultures.

Fig. 4. Effect of the CH_2Cl_2 extract on cell viability of NIH3T3 cells. NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium with various concentrations of the CH_2Cl_2 extract for 24 h. Cell viability was measured with the neutral red method. Results show means $\pm$ SD of three different experiments.

cell viability in NIH3T3 cells was examined by the NR method under similar conditions as the tumor cells. As shown in Fig. 4, the CH_2Cl_2 extract did not affect or decrease the cell viability of NIH3T3 cells.

Fig. 5. Dose-dependent effect of the CH_2Cl_2 extract on DNA synthesis of EATC. EATC were cultured with various concentrations of the CH_2Cl_2 extract for 5 h and then cultured in the presence of 50 μM bromodeoxyuridine (BrdU) for 19 h. The percentage of BrdU-positive cells was measured as the amount of DNA synthesis. Results show means $\pm$ SD of three different experiments. The differences between means were significant at ** $p < 0.01$ or * $p < 0.05$, compared with non- CH_2Cl_2 extract treated cultures.

Fig. 6. Effect of the CH_2Cl_2 extract on the cell cycle in EATC. EATC were cultured with the CH_2Cl_2 extract for 24 h. After fixation with 70% ethanol, the cells were incubated with freshly prepared propidium iodide-stained buffer for 1 h at 37°C in the dark. The cell cycle was analyzed by laser scanning cytometer. Results show mean $\pm$ SD of three different experiments. The differences between means were significant at ** $p < 0.01$ or * $p < 0.05$, compared with control cultures.

Effect of CH_2Cl_2 extract on DNA synthesis

The effect of various doses of the CH_2Cl_2 extract (0–100 $\mu\text{g/mL}$) on DNA synthesis was examined in EATC by the BrdU incorporation method. As shown in Fig. 5, the CH_2Cl_2 extract inhibited DNA synthesis significantly at low concentration (10 $\mu\text{g/mL}$) as compared with

Fig. 7. Dose- and time-dependent effect of the CH₂Cl₂ extract on Rb phosphorylation in EATC. In the dose-dependent study (A), cells were cultured in Eagle's minimum essential medium with various concentrations of the CH₂Cl₂ extract and harvested at 8 h. In the time-dependent study (B), cells were cultured with (+) or without (–) 200 µg/mL of the CH₂Cl₂ extract and harvested at indicated times. Cell lysis and Western blotting were performed as described under "Materials and Methods." ppRb: hyperphosphorylated Rb; pRb: hypophosphorylated Rb.

non-treated cells in a dose-dependent manner.

Effect of CH₂Cl₂ extract on cell cycle changes

The CH₂Cl₂ extract-induced cell death in EATC was also evaluated by cell cycle analysis with PI staining. The changes in cell cycle profile induced by CH₂Cl₂ extract at a 200 µg/mL concentration are shown in Fig. 6. Cells incubated with the CH₂Cl₂ extract of cape aloe resulted in an accumulation of the G1 cell cycle region and a decrease in the S and G2/M cell cycle region.

Effect of CH₂Cl₂ extract on Rb phosphorylation

The effect of CH₂Cl₂ extract treatment on Rb was measured by Western blot analysis. The CH₂Cl₂ extract decreased hyperphosphorylated Rb (ppRb) levels in EATC and increased hypophosphorylated Rb (pRb) levels in a dose- (Fig. 7A), and time-dependent manner (Fig. 7B).

DISCUSSION

The results obtained in the present study demonstrate for the first time, to the best of our knowledge, that CH₂Cl₂ extract of cape aloe caused a dose-dependent growth inhibitory effect (inhibition of cell viability and cell proliferation) in EATC, a decrease in DNA synthesis and an accumulation of cells in the G1 phase. This inhibitory effect is selective as the extract did not affect NIH3T3 cells used as a normal cell model. In addition, we showed that the growth inhibitory activities were derived from the decrease of hyperphosphorylated Rb.

The control of cell proliferation is crucial in maintaining cellular homeostasis and loss of this mechanism is a principal hallmark of cancer cells. Thus, the inhibition of tumor cell growth without side effects is recognized as an important target for cancer therapy. We have shown that CH₂Cl₂ extract of cape aloe inhibits cell

growth in EATC in a dose-dependent manner (Fig. 3). On the other hand, the CH₂Cl₂ extract did not induce cytotoxicity in NIH3T3 cell (Fig. 4). In addition, we investigated lactate dehydrogenase (LDH) release as an indicator of cell cytotoxicity (necrosis) in preliminary studies but CH₂Cl₂ extract of cape aloe did not show significant increase in LDH release in EATC (data not shown). These results suggest that cape aloe extract has cancer-specific selectivity and the growth inhibitory effect is not followed by cell injury (toxic).

Several factors regulated in the cell cycle have been extensively studied, and it has been shown that Rb and several cdk are involved in cell cycle progression (20, 21). Rb plays an important role in the control of progression through the G1 stage of the cell cycle (22–24). In early G1, hypophosphorylated Rb is present as a complex with the transcription factor E2F, thereby inactivating E2F. The phosphorylation of Rb in the mid-to-late G1 phase owing to the action of cdk-cyclin complexes, results in dissociation of the Rb-E2F complex and allows E2F to activate transcription of several genes, such as cyclin A, thymidine kinase and c-myc, which are required for progression through late G1 and into the S phase of the cell cycle (25).

In this study, CH₂Cl₂ extract of cape aloe inhibited cell proliferation and DNA synthesis due to an accumulation in the G1 phase (Fig. 6). In accordance with these results, the CH₂Cl₂ extract decreased hyperphosphorylated Rb (ppRb) levels and increased hypophosphorylated Rb (pRb) levels in dose- and time-dependent manners (Fig. 7).

Cape aloe contains a gel, consisting mainly of polysaccharide in a large amount of water fraction, as well as several chromones and their derivatives such as anthrone, anthraquinone glycoside and other phenolic compounds (26–28). The CH₂Cl₂ extract of cape aloe possibly contains some of these anthraquinone compounds, such as aloe-emodin and rhein. It was demonstrated that rhein, the active metabolite of sennoside laxatives, is a substrate for the glutathione-dependent multidrug resistance-associated protein 1 drug efflux pump and is a cytotoxic agent capable of inducing apoptosis (29). Aloe-emodin has been shown to cause cell death in human lung squamous cell carcinoma cell line CH27 (30) and in neuroectodermal tumors (31). Other investigators have also demonstrated that aloe-emodin inhibits cell proliferation and induces apoptosis in human hepatoma cell lines (32). Furthermore, it has been reported that aloe-emodin induces G2/M arrest of the cell cycle in human promyelocytic leukemia HL-60 cells (33). We measured the effect of aloe-emodin and rhein on cell viability in EATC in order to examine whether the effect of CH₂Cl₂ extract is due to aloe-emodin and rhein but neither compound decreased cell viability (data not shown). Therefore, it is not clear which components of the CH₂Cl₂ extract of cape aloe are responsible for the EATC cell death. The purification and identification of the effective compounds is ongoing in our laboratory.

With the exhibition of different effects on cancer and

normal cells observed by treatment with CH₂Cl₂ extract of cape aloe, possibly the first such study, we show that cape aloe possesses cancer-specific activity, and the growth inhibitory effect in EATC is related to changes in Rb phosphorylation. Consequently, we plan to use several animal models (in vivo) to elucidate and further clarify other mechanisms of the effect of cape aloe in EATC.

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