Inhibitory Effect of Propolis on the Growth of Human Leukemia U937

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We have investigated the effect of propolis (CB Propolis) on the growth of human histiolytic lymphoma U937 cells. We found that propolis strongly inhibited the growth of the cells and macromolecular synthesis in a dose- and time-dependent manner by apoptosis. Propolis at 0.015—0.5 μl/ml showed antitumor activity with an IC_{50} of 0.18 μl/ml for 3 d. It also inhibits DNA, RNA and protein synthesis with an IC_{50} of 0.08, 0.17 and 4.3 μl/ml, respectively. The inhibitory effect on DNA synthesis was partially irreversible. Moreover, an apoptotic DNA ladder and chromatin condensation were observed in the same concentration range in which cell growth was inhibited. The caspase inhibitor, Z-Asp-CH_2-DCB, prevented DNA fragmentation. These results suggest that the antitumor activity of propolis occurs through the induction of apoptosis. Propolis may be useful as a cancer chemopreventive and chemotherapeutic agent.

Key words propolis; growth-inhibitory effect; apoptosis; U937 cell

Propolis is a resinous material gathered by honeybees from the buds and bark of certain trees and plants. In Japan, propolis is widely used as a health food and the Japanese believe that it can cure inflammation, heart disease, and even diabetes and cancer. Biological properties of propolis have become a point of particular interest recently.[1)] The most used formulation in folk medicine is the ethanol extract.[2)] Chemical analysis indicated that propolis is a multicomponent mixture of various compounds with prevalence of flavonoids and phenolic acids.[3)] Several biological attributes such as anticancer, antioxidant, antimicrobial, anti-inflammatory and antibiotic activities have been reported for propolis.[4)] The standardization of propolis preparations is indeed difficult because of changes in chemical composition and pharmacological activities, resulting from variation in geographical and botanical origin.[5)] It is important to investigate their mechanisms of action in order to predict possible therapeutic and toxic effects, and to also use this information to develop and design new drugs that are even more effective for the prevention and treatment of cancer. We are interested in the effects of various natural products on cell growth in human cancer cells, as predictors of novel agents that may be useful in cancer chemoprevention or therapy.[6)] In this study, the inhibitory effects of propolis, a new preparation (CB Propolis) isolated from Brazilian propolis, on the growth of the human leukemia cell line U937 and on the synthesis of DNA, RNA and protein in U937 cells are discussed.

MATERIALS AND METHODS

Chemicals Aqueous solution of propolis,[7)] CB Propolis (the dried ethanol extract of Brazilian propolis), was obtained from a commercial supplier (ChatBlanc inc., Tokyo, Japan). Lyophilized CB Propolis is equivalent to 18.5 mg propolis per 100 μl. In this study propolis was diluted in dimethysulfoxide (DMSO) and filtered with a sterile filter prior to use, and then added at the appropriate final concentrations to cultures of U937 cells. Control cells were treated with the same amount of vehicle alone. The final DMSO concentrations never exceeded 0.5% (v/v). In this condition, we confirmed that apoptosis was not observed in U937 cells. Z-Asp-CH_2-DCB (a wide-spectrum caspase inhibitor) was purchased from Peptide Institute (Osaka, Japan). The cells were treated with 100 μM inhibitor for 1 h before the treatment with propolis. [^{3}H]thymidine (50 μCi/μmol), [^{3}H]uridine (55 μCi/μmol), and [^{3}H]leucine (60 μCi/mmol) were purchased from NEM Research Products (Boston, MA, U.S.A.). Other reagents used in this research were purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries Co. (Osaka) unless otherwise specified.

Cell Lines and Cell Culture To examine the effects of propolis on cell proliferation, U937 human histiocytic lymphoma cells were obtained from the Japanese Research Resources Bank, Tokyo, Japan. The cells (4×10^6 cells/ml) were grown in RPMI 1640 medium (Iwaki Co., Ltd, Tokyo) supplemented with 100 units/ml of penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum at 37 °C under a humidified 95% air 5% CO_2 atmosphere, and passaged every 7 d. U937 cells had a doubling time of about 24—30 h under these conditions.

Cell Proliferation Experiments Cells were inoculated at a density of 4×10^3 cells/well in 0.1 ml of medium using 96-well plates. One day later, to study the growth-inhibitory effects of propolis, cells were incubated with various concentrations of this material for 5 d. An appropriate volume of drug vehicle was added to untreated cells. After each period of incubation, aliquots of the cell suspensions were removed and the viable cells were counted using a trypan blue dye exclusion test for the appropriate period as previously described.[8)]

Measurement of DNA, RNA and Protein Synthesis in U937 Cells Cells were inoculated at a density of 4×10^3 cells/well in 0.1 ml of medium using 96-well plates. One day later, for measurement of DNA, RNA or protein synthesis, cells were placed in 96-well microtiter plates, and 0.5 μCi/ml [^{3}H]thymidine, 0.7 μCi/ml [^{3}H]uridine or 1 μCi/ml [^{3}H]leucine was added, respectively, to each individual plate followed by DMSO or propolis in DMSO. Plates were incubated at 37 °C for a given period, and the macromolecular substance was collected on glass fiber filters and washed three times with 5% cold TCA solution. The dry glass fiber filters were placed in scintillation vials with 10 ml of scintil-

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lation fluid and radioactivity was determined in a Beckman LS 6000TA scintillation counter.[9] The results were expressed as the percentage of the mean of duplicates incorporated in control cells incubated without propolis but otherwise treated in the same way.

Nuclear Morphologic Observation At appropriate times during the incubation, cells were collected and spun down at 2000 ***g*** for 5 min. The pellets were washed three times with ice-cold phosphate-buffered saline (PBS) and fixed for 30 min with 25% acetic acid in methanol. Then the cells were washed three more times with ice-cold PBS. After being resuspended, the cells were deposited on cyto-centrifuge slides and stained with 10 μl of 10 μM Hoechst 33258 (H 33258) for 30 min at room temperature. They were washed three times with ice-cold PBS and then observed through excitation and emission filters of 360 and 420 nm, respectively, with a fluorescence microscope, Eclipse TE 300 (Nikon, Tokyo). The number of apoptotic cells, defined as cells with condensed and fragmented nuclei, was evaluated on H 33258-stained preparations and expressed as a percentage of the total number of cells in untreated cultures.[10]

DNA Electrophoresis DNA fragmentation was analyzed by electrophoresis as described elsewhere.[10] After incubation with designated concentrations and schedules of propolis, 10^6 cells were pelleted. The genomic DNA was extracted with 1.4% agarose gel and visualized by ethidium bromide staining. The gel was photographed under UV fluorescence to detect qualitative damage to genomic DNA.

RESULTS AND DISCUSSION

Inhibitory Effect of Propolis on U937 Growth U937 cells were incubated with propolis at concentrations of 0.015, 0.05, 0.15 and 0.5 μl/ml for 5 d. To study the growth-inhibitory effects of propolis, cell proliferation was evaluated as a parameter of cytotoxicity. Propolis was not cytotoxic at any concentration after 8 h, and at a low concentration (0.015 μl) it had no effect on cell proliferation. At high concentrations (0.05, 0.15, 0.5 μl/ml), however, propolis abolished cell growth. Propolis inhibited the growth of U937 cells in a dose-dependent manner (Fig. 1), and its addition at 0.015, 05, 0.15, or 0.5 μl/ml and incubation at 37 °C for 3 d inhibited growth of the cells by 9, 25, 44, and 68%, respectively. The inhibitory effect of propolis on the growth of U937 cells was also dependent on the period of incubation. Incubation with this substance at 5 μl/ml at 37 °C for 1, 2, 3, 4, and 5 d inhibited the growth of U937 cells by 18, 45, 55, 63 and 60%, respectively.

Inhibitory Effect of Propolis on DNA, RNA and Protein Synthesis of U937 Cells The inhibitory effects of propolis on the synthesis of DNA, RNA and protein in U937 cells are shown in Fig. 2. The initial rates of incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine into trichloroacetic acid-insoluble material were utilized to estimate the rates of DNA, RNA and protein synthesis, respectively, in these cells. The presence of propolis at 0.015, 0.03, 0.05, 0.15, 0.3, 0.5, 1.5, 3, and 5 μl/ml in cultured U937 cells incubated for 60 min inhibited the incorporation of [^{3}H]thymidine into DNA by 16, 28, 37, 62, 85, 90, 100, 100, and 100% (Fig. 2), the incorporation of [^{3}H]uridine into RNA by 5, 18, 27, 54, 65, 72, 95, 90, and 92%, and the incorporation of [^{3}H]leucine into protein by 9, 15, 15, 21, 16, 34, 37, 40, and 47%, respectively. The results indicate that propolis is a potent inhibitor of DNA and RNA synthesis, but is somewhat less effective against protein synthesis in U937 cells. The inhibition by propolis of DNA synthesis in U937 cells occurred rapidly (data not shown), and the inhibition at 3 μl/ml was essentially completed after 30 min. The inhibitory effect of propolis on the DNA synthesis was dependent on the period of incubation. When U937 cells were incubated with propolis at 0.005, 0.015, 0.05, 0.15, 0.5, 1.5 and 5 μl/ml for 30 min at 37 °C, the DNA synthesis was inhibited by 8, 16, 20, 28, 80, 80, and 86%, respectively. An incubation of the cells with propolis at these same rates for 120 min at 37 °C, casued inhibition of the DNA synthesis was inhibited by 36, 40, 69, 73, 92, 98, and 99%, respectively. The inhibitory effect on DNA synthesis was partially irreversible (Fig. 3). U937 cells were preincubated with propolis at 0.005, 0.05 and 0.5 μl/ml for 60 min at 37 °C and then washed with phosphate-buffered saline (PBS) three times to

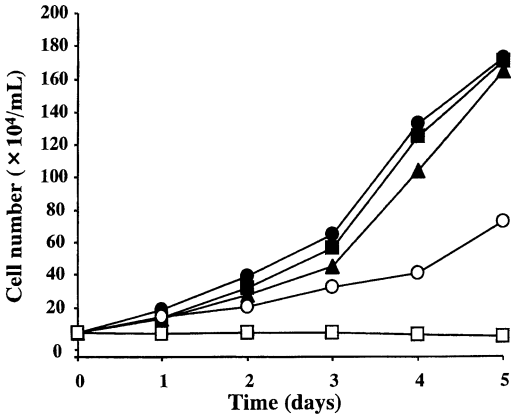

Fig. 1. Time- and Dose-Dependent Growth Inhibition of U937 Cells by Propolis

Cells (4×10^3 cells/well) were incubated in a 96-well microtiter plate for 5 d at 37 °C with propolis (0.015: ■, 0.05: ▲, 0.15: ○, and 0.5 μl/ml: □) and control (DMSO: ●) being added. The viable cells were counted every 24 h under a microscope using a trypan blue dye exclusion test. Each point represents the mean for 3 experiments. S.E.M. was usually within 10% of the mean value.

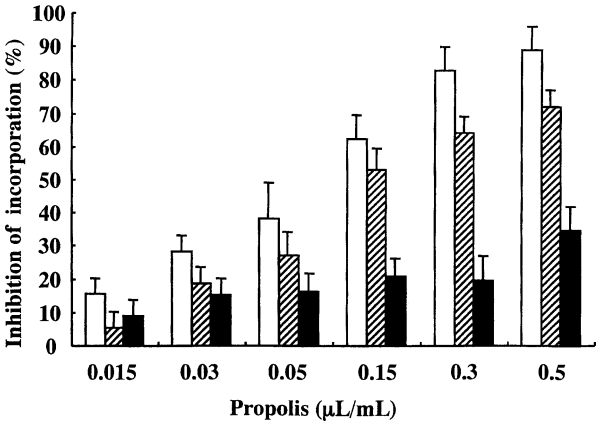

Fig. 2. Inhibitory Effect of Propolis on DNA, RNA and Protein Synthesis in U937 Cells

Cells were incubated with various concentrations of propolis (0.015—0.5 μl/ml) for 1 h, whereafter the incorporation of [^{3}H]-labeled thymidine (for monitoring DNA synthesis; □), uridine (for monitoring RNA synthesis; ▨) or leucine (for monitoring protein synthesis; ■) during the following hour was measured. Each point represents the mean of 3 experiments performed in triplicate. S.E.M. was usually within 10% of the mean value.

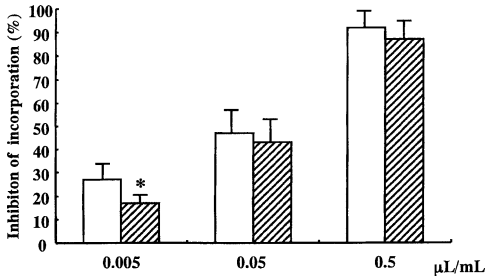

Fig. 3. Irreversible Inhibitory Effect of Propolis on the Synthesis of DNA in U937 Cells

□: Cells (4×10^4 cells/ml) were incubated with 2 μl DMSO at 37 °C for 60 min, were washed with PBS 3 times to remove the DMSO. Cells were resuspended in fresh medium and 2 μl of DMSO or propolis in DMSO and [^{3}H]thymidine was added, and incubated at 37 °C for 60 min. ▨: Cells (4×10^4 cells/ml) were preincubated with 2 μl DMSO, or propolis (0.005, 0.05, 0.5 μl/ml) in DMSO at 37 °C for 60 min, then washed with PBS 3 times to remove the propolis. Cells were resuspended in fresh medium, [^{3}H]thymidine was added, and incubated at 37 °C for 60 min. The radioactivity incorporated into TCA insoluble materials was measured. Each point represents the mean of 3 experiments performed in triplicate. Asterisks denote significance from corresponding control ($p<0.05$, Student's t-test).

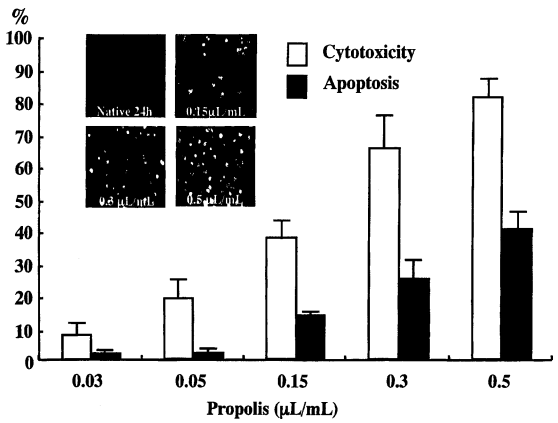

Fig. 4. Morphological Changes of U937 Cells Treated with Propolis

Cells were treated with propolis (0.03~0.5 μl/ml) or 2 μl DMSO for 24 h, then stained with 10 μl of 10 μM H 33258 for 30 min in the dark and the apoptotic cells (apoptotic-body formation) were counted under a fluorescence microscope. Results are presented as the mean±S.D. of 3 independent experiments. Apoptosis (Control: 0).

remove propolis. The cells were resuspended in medium, [^{3}H]thymidine was added and the DNA synthesis was evaluated. The results in Fig. 3 show that the inhibitory effect on DNA synthesis was dependent upon preincubation of the cells with concentrations of propolis. It is not known whether propolis can be removed by washing with PBS or whether it binds tightly to cells. The present investigation showed that propolis inhibited the growth of human leukemia U937 cells and the synthesis of macromolecules in these cells. The inhibitory effect on DNA synthesis was partially irreversible. These results suggest that some active components in propolis have antitumor activity. There is thus a possibility that propolis may be useful as a chemopreventive or chemotherapeutic agent.

Propolis-Induced Apoptosis Inhibition of apoptosis is one mechanism of tumor formation, several chemotherapeutic compounds have been reported to induce apoptosis and this may be the primary mechanism for their antitumor activity.[11,12] We therefore hypothesized that the induction of apoptosis was involved in the antitumor activity of propolis. To test this hypothesis, we first investigated the effect of propolis on the morphological changes to U937 cells. One of the most characteristic features of apoptosis is DNA degradation and the formation of a DNA ladder due to internucleosomal cleavage of chromosomal DNA.[13]

Cells incubated in the presence or absence of propolis at 37 °C for 24 h were stained with H 33258 and chromatin condensation was examined under a fluorescence microscope with excitation at 365 nm. As shown in Fig. 4, propolis treatment was confirmed to have an effect by the observation of chromatin condensation, a morphological change characteristic of apoptosis,[13] in the same concentration range at which it inhibited cell growth. Percentages of cells with chromatin condensation were calculated from three randomly selected fields of view. The percentages (means) of the number of apoptotic cells with chromatin condensation were 2.1, 2.3, 16.3, 27.3 and 40.1% in the presence of propolis at 0.03, 0.05, 0.15, 0.3 and 0.5 μl/ml, respectively, showing the concentration-dependence of the activity. Consequently, we conclude that propolis induced apoptosis in U937 cells. To investigate whether DNA fragmentation induced by

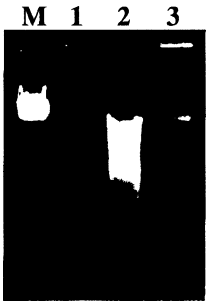

Fig. 5. Agarose Gel Electrophoresis of DNA Extracted from U937 Cells Treated with Propolis in the Presence of Caspase Inhibitor

Cells were treated with 2 μl DMSO (lane 1), propolis (0.1 μl/ml; lane 2), and propolis and Z-ASP-CH_2-DCB (lane 3). After 24 h culture, cells were collected and used for detection of fragmented DNA. *Hae*-III-digested ϕX-174 DNA fragments were used as molecular markers (Lane M).

propolis was the result of apoptosis, we performed agarose gel electrophoresis of DNA extracted from U937 cells that had been treated with propolis. As shown in Fig. 5, an apoptotic DNA ladder was observed in U937 cells when treated with the material. It is well known that activation of the cascade composed of various caspases occurs in apoptosis signal transduction and execution.[14] Therefore, we examined whether the cascade of caspases participated in the apoptosis induced by propolis. We investigated the effect of a caspase inhibitor on the DNA fragmentation induced by propolis and found that, Z-Asp-CH_2-DCB, which is known as a wide-spectrum caspase inhibitor, completely prevented the induced DNA fragmentation (Fig. 5). We also examined whether Z-Asp-CH_2-DCB blocked the morphological change of the apoptosis induced by propolis (data not shown). These findings are not limited to U937 cells, since similar results have been obtained with the J774.1, P388, HL-60 and Jurkat leukemia lines (data not shown). Consequently, the activity of caspases did participate in the apoptosis induced by propolis. Our results suggest that propolis induced apoptosis *via* caspase pathways. It would be interesting to investigate what the death substrates are which are cleaved by caspases and to clarify which caspases play a crucial role in the apoptosis induced by propolis. Moreover, we want to investigate the death mechanism for the activation of caspases and the

execution of apoptosis.

A preliminary study has indicated that propolis inhibits the growth of U937 cells through induction of apoptosis. Propolis may be useful as a cancer chemopreventive and chemotherapeutic agent.

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REFERENCES AND NOTES

1) Walker P., Crane E., *Apidologie*, **18**, 327—334 (l987).
2) Menezes H., Alvaarez J. M., Almerida E., *Arzneim Forsch/Drug Res.*, **49**, 705—707 (l999).
3) Greenway W., May J., Scaysbrook T., Whatley F. R., *Zeitschrift für Naturforschung*, **46c**, 111—121 (l991).
4) Nagai T., Inoue R., Inoue H., Suzuki N., *Food Chem.*, **80**, 29—33 (2003).
5) Ghisalberti E. L., *Bee World*, **60**, 59—84 (l997).
6) Kanno S., Shouji A., Asou K., Ishikawa M., *J. Pharmacol.*, **92**, 166—177 (2003).
7) Propolis extract is a commercial product consisting of propolis with out waxes; it is usually obtained from propolis by alcohol extraction and normally called “propolis.”
8) Ishikawa M., Fujita R., Furusawa S., Takayanagi M., Sasaki K., Satoh S., *Biol. Pharm. Bull.*, **24**, 1185—1187 (2001).
9) Kanno S., Ishikawa M., Takayanagi M., Takayanagi Y., Sasaki K., *Biol. Pharm. Bull.*, **23**, 37—42 (2000).
10) Kanno S., Ishikawa M., Takayanagi M., Takayanagi Y., Sasaki K., *Biol. Pharm. Bull.*, **22**, 1296—1300 (1999).
11) LaCasse E. C., Baird S., Korneluk R. G., Mackenzie A. E., *Oncogene*, **17**, 3247—3259 (l998).
12) Kaufmann S. H., Earnshaw W. C., *Exp. Cell Res.*, **256**, 42—49 (2000).
13) Darzynkiewicz Z., Li X., Gong J., *Methods Cell Biol.*, **41**, 1—38 (1995).
14) Grutter M. G., *Curr. Opin. Struct. Biol.*, **6**, 649—663 (2000).