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Inhibition of Thymocyte Apoptosis by Berberine

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ABSTRACT. To find anti-apoptotic substances in plant resources, a microassay method for estimating DNA fragmentation was established using fluorochrome 3,5-diaminobenzoic acid dihydrochloride. Examination was made of various herbal medicines for inhibitory effects on glucocorticoid-induced apoptosis in thymocytes. Several Kampo medicines, e.g. Oren-gedoku-to and San'o-shashin-to, were found to inhibit dexamethasone-induced apoptosis in murine thymocytes. Some of these medicines contain *Coptidis rhizoma* (CR) as the major constituent, and the CR extract showed the most potent inhibitory activity on thymocyte apoptosis of more than 200 species of herbal extracts. The inhibition of apoptosis by CR extract was confirmed by the trypan blue exclusion test, lactate dehydrogenase release measurement, and morphological evaluation by electron microscopy. The benzodioxolo-benzoquinolizine alkaloid, berberine, and five berberine-type alkaloids, isolated from CR extract, had an inhibitory effect, whereas no effect was noted for the aporphin-type alkaloid magnoflorine. The inhibitory action of berberine was also demonstrated on etoposide- and camptothecin-induced apoptosis. BIOCHEM PHARMACOL 53;9:1315–1322 © 1997 Elsevier Science Inc.

KEY WORDS. apoptosis; inhibitor; thymocyte; berberine; *Coptidis rhizoma*; 3,5-diaminobenzoic acid dihydrochloride

Apoptosis is essential to a wide variety of physiological processes [1–4]. It occurs in numerous cell types in response to various physiological and pathological stimuli. The induction of apoptosis in any tissue is strictly regulated, so that the mechanism of cell death can only be interfered with by a particular tissue-specific signal. Investigation of specific inhibitors of an apoptotic system should provide additional insight into the mechanisms of cell-type- or tissue-specific regulation of apoptosis and the physiological roles of apoptosis in tissue homeostasis.

Plants abundantly contain bioactive materials that interfere with signal transduction and cell metabolism pathways. Several compounds of plant origin have modulating effects on apoptosis; quercetin induces apoptosis in K562, Molt-4, Raji, and MCAS tumor cells [5]; camptothecin and taxol are anticancer drugs that effectively induce apoptosis in many tumor cells [6–8]; genistein, a protein kinase inhibitor, induces or inhibits apoptosis in various cells [8–13]; and baicalin induces apoptosis in several lines of hepatoma cells [14] and HIV-infected T lymphocytes [15] but has no effect on the viability of normal hepatocytes or lymphocytes. Primary and secondary metabolites in plants may function as synergistic and broad-spectrum antioxidants [16]. Oxidative stress is important in apoptosis not only as an apoptotic stimulus but also as an intracellular mediator of apoptotic signal transduction pathways [17, 18]. Thus, plant-derived metabolites may function to inhibit apoptosis. Investigation of apoptosis-modulating agents in plant resources should help elucidate the molecular mechanisms of apoptosis.

Hence, the authors have screened a few hundred herbal extracts and Japanese herbal medicines (Kampo medicines) for inhibitory effects on *in vitro* experimental systems of apoptosis and found the aqueous extract of *Artemisiae capillaris spica* and its ingredients to exert a potent inhibitory effect on hepatocyte apoptosis [19]. In the present study, the authors established a microassay method for assessing the extent of glucocorticoid-induced apoptosis in thymocytes and investigated various herbal medicines for inhibitory effects on this model. Berberine and berberine-type alkaloids, produced in numerous plants of genera *Berberis* and *Coptis*, potently inhibited thymocyte apoptosis. The action was also found toward etoposide- and camptothecin-induced apoptosis.

MATERIALS AND METHODS

Drugs and Reagents

Extract powders of medicinal plants and Japanese traditional medicines (Kampo medicines) were prepared by

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Tsumura & Co. (Tokyo, Japan). Each Kampo medicine was obtained as an extract powder from a mixture of medicinal herbs according to the traditional prescriptions. For example, OGT** extract powder consists of crude ingredients extracted from the following four medicinal herbs mixed in the ratios in parentheses; *Scutellariae radix* (3.0), *Coptidis rhizoma* (2.0), *Gardeniae fructus* (2.0), and *Phellodendri cortex* (1.0). Mixed medicinal herbs were extracted with boiling water, and the resulting aqueous extracts were spray-dried to obtain extract powders. The Kampo medicines manufactured by Tsumura & Co. have been approved as ethical drugs by the Ministry of Health and Welfare of Japan. The extracts of medicinal herbs and six ingredients isolated from CR, (columbamine, palmatine, epiberberine, groenlandicine, jateorrhizine and magnoflorine) were provided by the Phytochemistry Department and Natural Product Chemistry Department of the Central Research Laboratories of Tsumura & Co. Each extract powder was weighed and used by dissolving in culture medium at the desired concentration.

Berberine chloride, DEX, and SDS were obtained from Wako Chemicals (Osaka, Japan). DABA was obtained from the Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) and trypan blue were purchased from GIBCO-BRL (Gaithersburg, MD, U.S.A.). [^{3}H]Thymidine was obtained from Amersham Japan (Tokyo, Japan). The other chemicals were from Sigma (St. Louis, MO, U.S.A.) unless otherwise stated.

Cell Culture

Thymocytes were obtained routinely from C3H/HeN mice (male 3- to 6-weeks-old) purchased from Japan SLC Inc. (Shizuoka, Japan). The thymocytes were suspended in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 10% heat-inactivated FBS and then filtered through a 75-μm mesh. The cells were plated in a 24-well plate, a 6-well plate (Nunclon, Denmark), a 96-well microtiter plate (Coster, Cambridge, MA, U.S.A.) and a 100 mm tissue culture dish (Corning, NY, U.S.A.), and maintained at 37° in 5% CO_2.

All experiments were conducted according to institutional guidelines for the care and use of laboratory animals in research.

Induction of Apoptosis and Quantitation of DNA Fragmentation

The authors developed a new microassay method for assessing DNA fragmentation in thymocytes. Freshly isolated thymocytes were seeded at a density of 6×10^6 cells/mL in 96-well microplates. The wells for total DNA estimation were processed immediately for cell lysis and quantitation of DNA. The experimental wells received the inducers (DEX, 10^{-7} M; etoposide, 10^{-5} M; or camptothecin, 10^{-5} M, respectively) and/or test samples and then were incubated for 6–24 hr before further processing. The cells were lysed by 1/10 vol. 50 mM Tris–HCl (pH 8.0)/200 mM EDTA/5% Triton X-100. The plates were centrifuged at 750 g, 4° for 16 hr, and formaldehyde was added to a final concentration of 10%. Following decantation of the supernatant and several washings in tap water, DNA in the pellet at the bottom of the well was quantitated by fluorometric assay using DABA, which reacts with deoxyribose sugars exposed after the removal of purine bases by hot acid hydrolysis [20, 21]. The plate was dried, and 100 μL of 0.4 g/mL DABA solution was added. After incubation for 2 hr at 55°, fluorescence was determined by a Titertek Fluoroscan (Labsystems, Helsinki, Finland) equipped with an excitation filter of 400 nm and an emission filter of 500 nm. DNA was estimated from the calibration curve separately prepared. The percentage of DNA fragmentation induced by the reagents was determined as:

$$\text{DNA fragmentation (\%)} = \frac{\text{(total DNA amount)} - \text{(DNA amount in the experimental wells)}}{\text{total DNA amount}} \times 100$$

Viability Assay

Viability of the cultured cells was determined by trypan blue exclusion. Cell membrane integrity was assayed based on LDH activity released into the culture medium in the LDH-Cytotoxic Test (Wako Chemicals). The percentage of LDH released was defined as the ratio of LDH activity in the supernatant to that in the lysate of a whole cell suspension.

DNA Gel Electrophoresis

To prepare DNA for agarose gel electrophoresis, cells (3×10^6) were collected by centrifugation and suspended in 10 mM Tris–HCl, pH 8.0, 0.1 M EDTA, 0.5% SDS and incubated with RNase A (20 μg/mL) for 1 hr at 37° and then with proteinase K (100 μg/mL) for 16 hr at 37°. Following additional incubation for 3 hr at 50°, the crude DNA mixture was electrophoresed on a 2% agarose gel containing 0.5 μg/mL ethidium bromide and visualized by UV illumination.

Electron Microscopy

For electron microscopic examination, aliquots of thymocyte suspension were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and then post-fixed with 1% osmium tetroxide in the same buffer and embedded in epoxy resin, Quetol-812 (Nisshin EM Co. Ltd., Tokyo). Ultrathin sections were double-stained with uranyl acetate

** Abbreviations: CR, *Coptidis rhizoma*; OGT, Oren-gedoku-to; SST, San'o-shashin-to; DEX, dexamethasone; CHX, cycloheximide; DABA, 3,5-diaminobenzoic acid dihydrochloride; and LDH, lactate dehydrogenase.

and lead citrate and observed under a JEM-200CX (JEOL Co. Ltd., Tokyo).

RESULTS

DEX-treated thymocytes showed a typical DNA ladder formation, a certain indication of apoptosis, the degree of which can be evaluated by quantitating DNA fragmentation. We preliminarily confirmed the value of percent DNA fragmentation in the microassay procedure to be essentially the same as that by other conventional DNA fragmentation assays using diphenylamine [22–24] (data not shown). DEX showed concentration- and time-dependent induction of DNA fragmentation in thymocytes (Fig. 1, A and B, respectively). Maximal induction of apoptosis by DEX occurred at concentrations higher than 5×10^{-9} M. When freshly isolated mouse thymocytes were incubated in 10^{-7} M DEX, DNA fragmentation became evident after incubation for 3 hr, and at the end of the culture time (18 hr) fragmented DNA had increased to 92.4%, while fragmented DNA in control cultures was less than 24.1%.

By this assay system, we examined the degree of apoptosis in thymocytes treated with DEX in the presence or absence of 126 Kampo medicines at 200 μg/mL. As evident from Fig. 2, OGT and SST inhibited DEX-induced DNA fragmentation in thymocytes in a concentration-dependent manner. Other Kampo medicines showed less potent or no inhibitory effect on cell death. Next, we screened aqueous extracts of over 200 species of medicinal herbs at 100 μg/mL. The aqueous extract of CR showed the most potent inhibitory effect. The effect of CR extract was concentration dependent, and the extract completely suppressed DNA fragmentation at 60 μg/mL (Fig. 3). OGT and SST contain CR as a constituent herb, suggesting that the inhibitory effects of these Kampo medicines may be due to the effect of CR.

The inhibition of thymocyte apoptosis by the CR extract was examined by several criteria. The results of agarose gel electrophoresis of DNA extracted from DEX-treated thymocytes in the absence and presence of CR extract are shown in Fig. 4. The appearance of the DNA ladder pattern was suppressed by addition of the CR extract (50 μg/mL). Trypan blue exclusion and LDH release demonstrated the integrity of the cell membrane to be lost as a result of DEX treatment for 24 hr, although in the presence of the CR extract, the cells remained viable (Table 1). The difference between the data on cell viability assessed by trypan blue exclusion and LDH release probably originates from the difference in their sensitivity for detecting loss of the cell membrane integrity. However, both results showed a similar tendency in the inhibitory effect of CHX and CR on decrease of viability of DEX-treated thymocytes. The CR extract thus suppresses early and late apoptotic changes in thymocytes. We have also analyzed the morphology of CR/DEX-treated cells by electron microscopy (Fig. 5, A–D). DEX-treated cells displayed typical apoptotic mor-

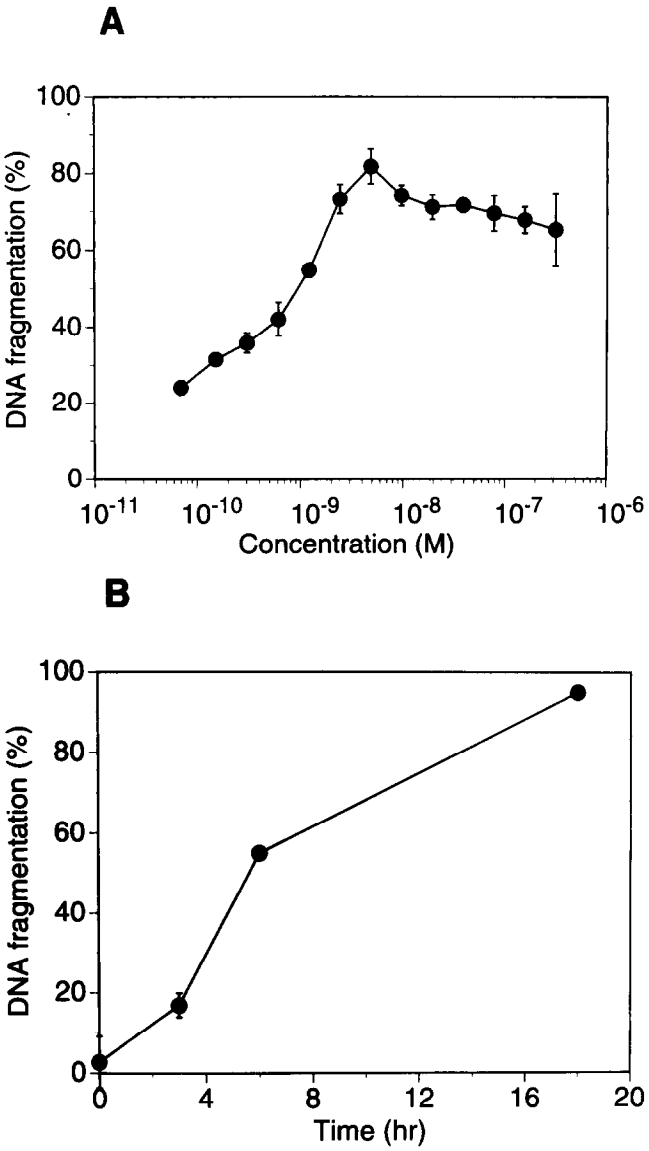

FIG. 1. DNA fragmentation induced by DEX in thymocytes. (A) Concentration-dependency of DEX-induced DNA fragmentation. Cells were incubated for 18 hr in the presence of various concentrations of DEX. DNA fragmentation was measured as described in Materials and Methods. Values represent means ± SD (N = 3). The data shown are representative of three independent experiments with similar results. (B) Time–course of DEX-induced DNA fragmentation. Cells were treated for various times with 10^{-7} M DEX. DNA fragmentation was measured as described in Materials and Methods. Values represent means ± SD (N = 3). The data shown are representative of three independent experiments.

phology, including reduced cell size and nuclear condensation, after a 6-hr incubation (Fig. 5B). In contrast, thymocyte cultures coincubated with DEX and the CR extract remained morphologically normal (Fig. 5D).

To explain the inhibitory effects of the CR extract, several ingredients of CR were investigated for inhibitory effects on thymocyte apoptosis. A benzodioxolo-benzoquinolizine alkaloid, berberine, a major component of

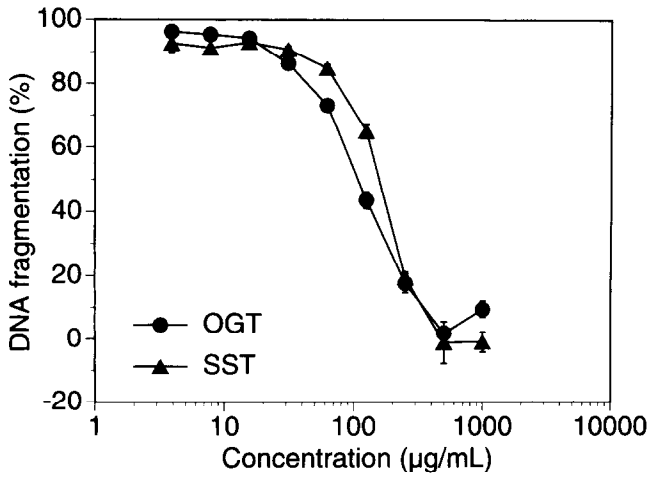

FIG. 2. Concentration-dependent inhibition of DEX-induced DNA fragmentation by OGT and SST. Cells were treated for 18 hr with 10^{-7} M DEX in the presence of various concentrations of each Kampo medicine. DNA fragmentation was measured as described in Materials and Methods. Values represent means ± SD (N = 6). The data shown are representative of two independent experiments.

CR, concentration-dependently inhibited DNA fragmentation (Fig. 6, A and B). A study was made to determine whether six compounds isolated from CR, i.e. columbamine, palmatine, epiberberine, groenlandicine, jateorrhizine, and magnoflorine, would protect thymocytes from DEX-induced DNA fragmentation. Berberine-like alkaloids (columbamine, palmatine, epiberberine, groenlandicine, and jateorrhizine) had suppressive action, whereas the aporphin-type alkaloid magnoflorine in this study did not (Fig. 7). The inhibitory activity on thymocyte apoptosis would

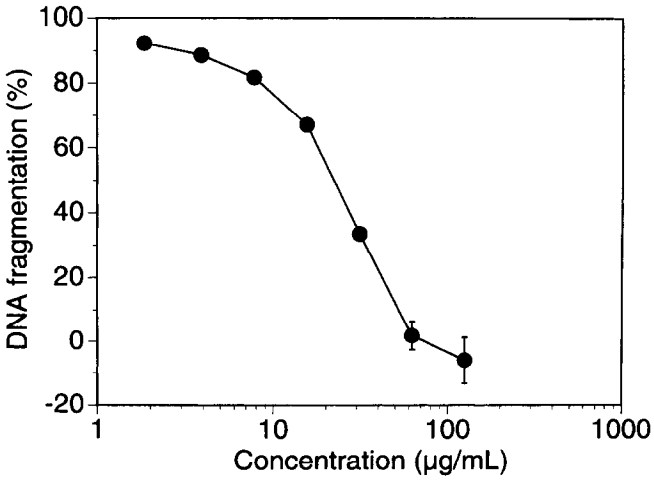

FIG. 3. Concentration-dependent inhibition of DEX-induced DNA fragmentation by CR extract. Cells were treated for 18 hr with 10^{-7} M DEX in the presence of various concentrations of CR extract. DNA fragmentation was measured as described in Materials and Methods. Values represent means ± SD (N = 6). The data shown are representative of five independent experiments.

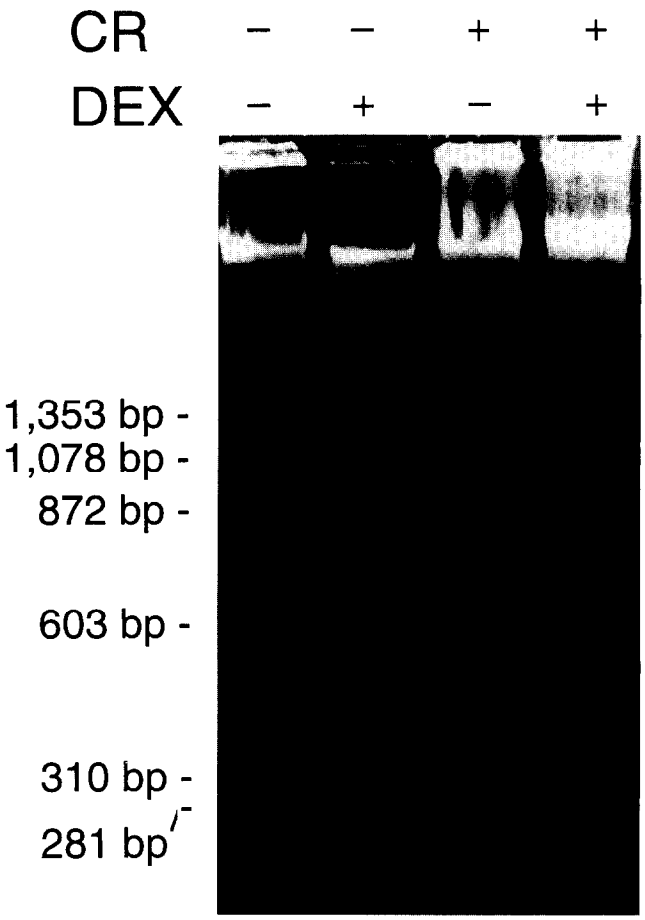

FIG. 4. Agarose gel electrophoresis of DNA extracted from CR-treated/untreated thymocytes. Cells were treated for 6 hr with 10^{-7} M DEX in the presence of 50 μg/mL of the CR extract. Cells (3×10^6) were digested with RNase A and proteinase K as described in Materials and Methods, and the crude DNA mixtures were electrophoresed on a 2% agarose gel.

thus appear to be a common feature of berberine-like substances.

Finally, we examined the effect of berberine on anticancer drug-induced apoptosis in thymocytes. Etoposide and camptothecin induced apoptosis in thymocytes at 10^{-5} M.

TABLE 1. Effects of CR extract on viability: LDH release and trypan blue exclusion test

	Viability (%)	
Treatment	**LDH**	**Trypan blue**
None	38.9 ± 15.9	9.6 ± 8.3
CHX (12.5 ng/mL)	88.1 ± 1.8	69.0 ± 12.3
CR (50 μg/mL)	102.8 ± 1.4	45.8 ± 9.6

Thymocytes were seeded at a density of 6×10^6 cells/mL in 96-well microplates and incubated with 10^{-7} M DEX in the presence or absence of CR or CHX at concentrations indicated in the table. LDH release and trypan blue exclusion were determined at 24 hr as described in Materials and Methods. The CR extract and CHX, a well-known inhibitor of thymocyte apoptosis, showed suppressive activity on the DEX-induced loss of viability. Data are means ± SD (N = 3).

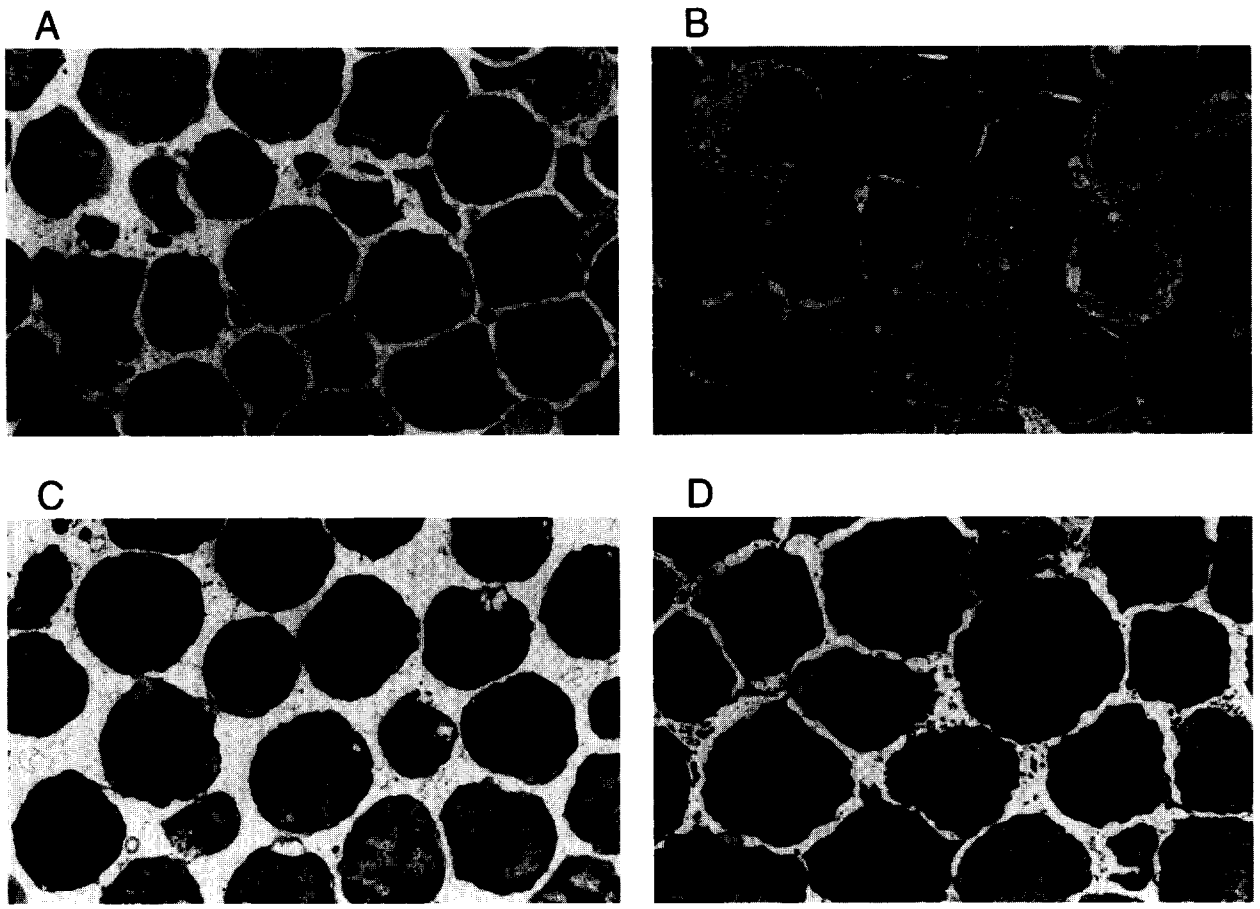

FIG. 5. Morphological examination of CR-treated thymocytes by electron microscopy. Cells were incubated for 6 hr in control cultures (A), in the presence of 10^{-7} M DEX (B), 50 μg/mL of CR (C), or 10^{-7} M DEX and 50 μg/mL of CR (D). Apoptotic cells (an arrow in B) were characterized by chromatin condensation. (Original magnification: 4000×.)

The coincubation of 100 μg/mL berberine, CR extract, and OGT markedly inhibited etoposide- and camptothecin-induced apoptosis (Table 2).

DISCUSSION

To find anti-apoptotic substances in plant metabolites, simple assay systems are needed to facilitate the processing of a large number of samples. For spontaneously proliferating cells, a microassay system for the quantitation of DNA fragmentation using DNA labeling by [^{3}H]thymidine incorporation and harvesting devices [19] was reported previously. In the present study, we developed a new microassay method for spontaneously nonproliferating cells for which [^{3}H]thymidine-labeling techniques are not applicable. This assay was an adaptation of the standard DNA fragmentation assay to the 96-well microtiter format, and DNA quantitation by the conventional diphenylamine procedure was replaced by the DABA fluorometric assay for greater sensitivity. The results obtained were similar to those of the standard DNA fragmentation assay.

Using these systems, we have screened herbal extracts and Kampo medicines for inhibitory effects on several apoptosis models, e.g. transforming growth factor-β1-mediated apoptosis in hepatocytes and Morris hepatomas, Fas-mediated apoptosis in several cell lines from different lineages, and glucocorticoid- and anticancer drug-induced apoptosis in thymocytes. This study demonstrates that the CR extract and CR-containing drugs have potent inhibitory effects on DEX-induced apoptosis in murine thymocytes. The anti-apoptotic activity of CR extract appears limited to thymocyte apoptosis, since the extract showed no or only a weak inhibitory effect on apoptosis in various apoptosis models using hepatocytes, several hepatomas, and the human promyelocytic leukemia cell line HL-60 (data not shown). We previously reported the aqueous extract of *Artemisiae capillaris spica* to exhibit the most potent inhibitory activity on apoptosis in Morris hepatoma McA-RH8994 cells, rat hepatocytes [19], and several hepatomas, while the extract had no effect on apoptosis in non-hepatic cells such as murine thymocytes, several human T cell lines, and HL-60 cells (unpublished observation). The potency of the inhibitory effect of a particular herb extract would thus appear to depend on cell type. The present findings are in accord with the multiplicity and diversity of apoptotic signal pathways already demonstrated in the literature [25].

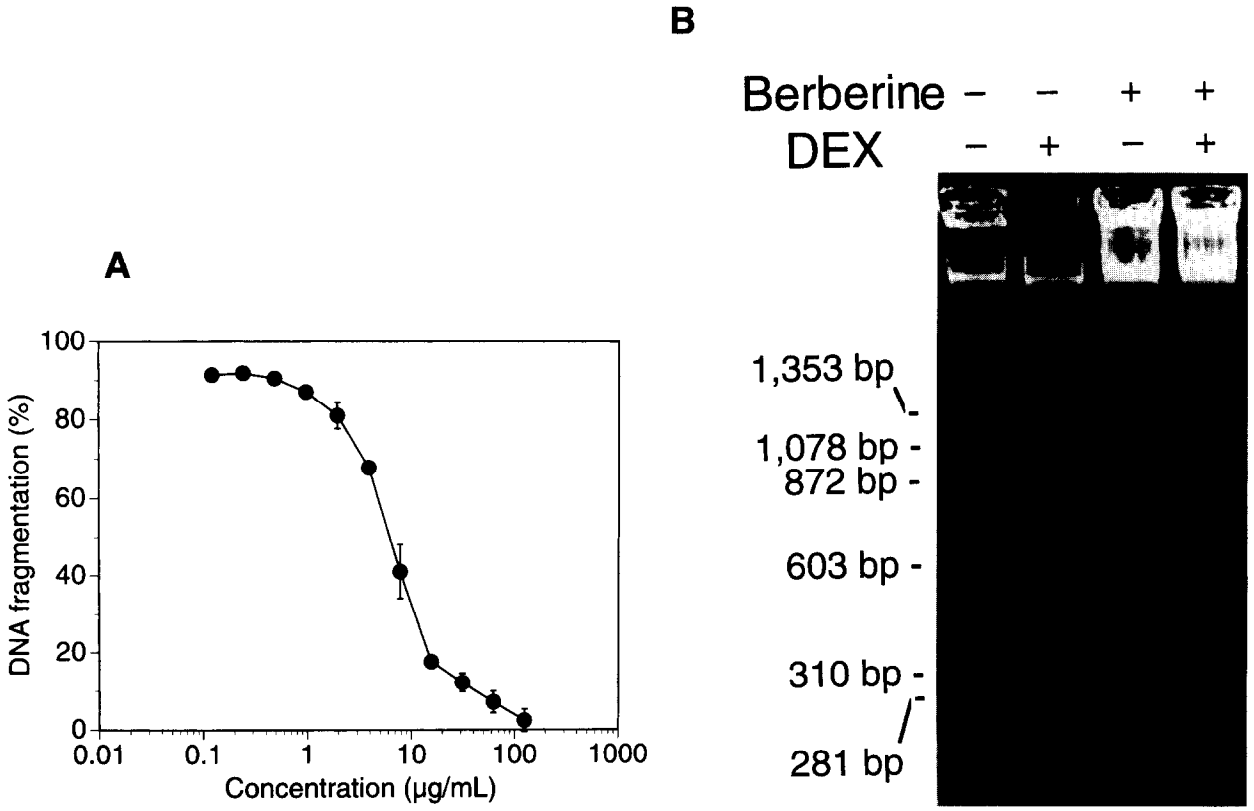

FIG. 6. Inhibition of DEX-induced DNA fragmentation by berberine. (A) Cells were treated for 24 hr with 10^{-7} M DEX in the presence of various concentrations of berberine. DNA fragmentation was measured as described in Materials and Methods. Values represent means ± SD (N = 6). The data shown are representative of two independent experiments with similar results. (B) Agarose gel electrophoresis of DNA extracted from berberine-treated/untreated thymocytes. Cells were treated for 6 hr with 10^{-7} M DEX in the absence or presence of 20 μg/mL of berberine. DNA electrophoresis was performed as described in the legend of Fig. 4.

Berberine is a benzodioxolo-benzoquinolizine alkaloid present in numerous plants of the genera *Berberis* and *Coptis*, which for centuries have been used in Europe and Far Eastern countries for treating gastroenteritis and diarrhea. It has a wide range of pharmacological and biological activities, including antisecretory [26–30], anti-inflammatory [31], antimicrobial [32–36], and anticancer properties [32, 37]. Berberine expresses cytotoxic activity toward several tumor cells [38] and induces apoptosis in HL-60 cells [39]. The present study shows berberine to inhibit apoptosis in thymocytes exposed to DEX, etoposide and camptothecin. This is the first demonstration, as far as we know, that berberine and berberine-like alkaloids have an ability to inhibit apoptosis.

Anticancer drugs induce apoptosis in many tumor cells via interactions with DNA. The strong binding of berberine to DNA has been reported [40–43], and the anticancer and antibacterial effects of berberine are considered to be related to interactions with DNA [43]. Kuo *et al.* [39] found berberine to induce apoptosis in HL-60 cells and DNA in berberine-treated cells to be closely associated with this alkaloid. However, they noted that palmatine, an analog of berberine, does not induce apoptosis in HL-60 cells, in spite of the tight binding of palmatine to DNA. In the present

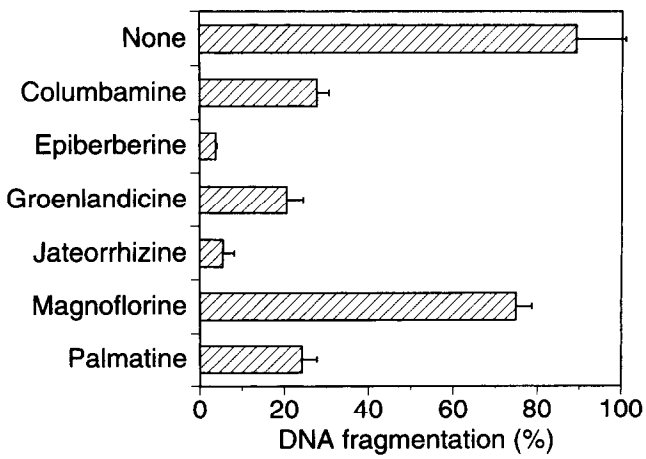

FIG. 7. Effects of various ingredients isolated from CR on DEX-induced DNA fragmentation in thymocytes. Cells were incubated for 16 hr in the presence of 10^{-7} M DEX and 200 μg/mL of each ingredient and assayed for DNA fragmentation as described in Materials and Methods. Values represent means ± SD (N = 6).

TABLE 2. Effects of OGT, CR extract, and berberine on anticancer-drug-induced apoptosis

Treatment		DNA fragmentation (%) Etoposide	Camptothecin
None		81.8 ± 3.1	82.6 ± 1.4
CHX	(12.5 ng/mL)	31.8 ± 2.5	30.0 ± 1.2
OGT	(100 μg/mL)	56.0 ± 4.3	57.8 ± 2.0
CR	(100 μg/mL)	43.6 ± 2.7	44.1 ± 4.5
Berberine	(100 μg/mL)	38.6 ± 8.3	52.5 ± 8.2

Thymocytes were incubated with 10^{-5} M etoposide or 10^{-5} M camptothecin in the presence or absence of OGT, CR, berberine, or CHX at concentrations indicated in the table. DNA fragmentation was measured as described in Materials and Methods. Data are means ± SD (N = 6). CHX was used for comparison.

study, berberine and berberine-like alkaloids including palmatine exhibited similar inhibitory effects on thymocyte apoptosis. Thus, binding to DNA should not be lethal by itself, and other cellular processes are required for berberine to elicit a cytotoxic or cytoprotective effect.

Berberine has been reported to inhibit DNA, RNA, and protein synthesis in S180 tumor cells [32] and 9L rat glioma cells [38]. Inhibitors of macromolecular synthesis, such as actinomycin D and CHX, suppress thymocyte apoptosis, apparently by inhibiting the biosynthesis of "killer proteins," supposed to be components of the cell death mechanism [44, 45]. The anti-apoptotic activity of berberine may thus be attributable to its inhibitory activity toward protein synthesis. However, recent studies have revealed the necessity of the re-evaluation of the role of *de novo* protein synthesis in apoptosis. For instance, CHX induces and blocks apoptosis in response to a determined stimulus in the same cell type [46–48]. The protein synthesis inhibitor puromycin induces apoptosis in rat thymocytes, and CHX blocks puromycin-induced apoptosis [49]. Our preliminary observations suggest that berberine inhibits apoptosis in some T cell lines that cannot be blocked by the inhibitors of macromolecular synthesis.*

It should be remembered that the inhibition of apoptosis does not necessarily translate into an increased survival of cells. Lock and Stribinskiene [50] recently reported that bcl-2-expressing HeLa cells showed a marked inhibition of etoposide-induced apoptosis, but no significant increase in survival when assessed by a colony-forming assay. In our study, the inhibition of apoptosis in DEX-treated thymocytes was confirmed using several criteria, but the functional integrity of treated thymocytes is still in question. It may be important to clarify not only the effect of berberine on apoptosis, but also its effect on the functional state of the thymocyte, to understand the potential implications of our findings in the development of drugs.

Herbal extracts consist of many kinds of ingredients, and their biological activity is usually not attributed to a single moiety. In this study, we found that many kinds of berberine-like alkaloids inhibit DEX-induced thymocyte apoptosis. The degree of activity may differ among the alkaloids, and therefore future investigation of the structure–activity relationship may provide information for the development of inhibitors more active against thymocyte apoptosis. Modulation of thymocyte apoptosis may have potential implications for the regulation of the immune system, which may provide new means for the treatment of immunological disorders. The elucidation of the mechanisms by which berberine exerts its inhibitory effect on apoptosis, especially the target molecule of berberine in the machinery of apoptosis, is also important for this reason and requires further investigation. These studies are now in progress in our laboratory.

* Manuscript in preparation.

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