Jpn. J. Cancer Res. 85, 645–651, June 1994

Selective Inhibitory Effect of Bufalin on Growth of Human Tumor Cells *in vitro*: Association with the Induction of Apoptosis in Leukemia HL-60 Cells

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We found that bufalin, an active principle of the Chinese medicine *chan'su*, has selective inhibitory effects on the growth of various human cancer cells. In order to examine whether the growth-inhibitory effect of bufalin on human cancer cells is associated with apoptosis, human leukemia cells were treated with bufalin. HL-60, ML1, and U937 leukemia cells treated with bufalin at 10^{-8} *M* and above had condensed and fragmented nuclei. Flow cytometric analysis of these cells treated with bufalin showed fragmented DNA smaller than that of the G1 phase. DNA of HL-60 cells treated with bufalin showed a ladder pattern characteristic of apoptosis, as analyzed by agarose gel electrophoretic analysis. DNA synthesis and topoisomerase II activity of HL-60 cells were markedly inhibited as the concentration of bufalin was increased. The concentration needed for inducing apoptosis of HL-60 cells was 10^{-8} *M*, which is comparable to that of camptothecin, but lower than those of other antitumor drugs such as cisplatin, VP16 and all-*trans* retinoic acid. Apoptosis was not observed when human mononuclear and polymorphonuclear cells were treated with 10^{-6} *M* bufalin for 24 h. These results indicate the association of the growth-inhibitory effect of bufalin with the induction of apoptosis, at least in HL-60 cells, and suggest the usefulness of bufalin for differentiation-apoptosis-inducing therapy for cancer.

Key words: Bufalin — Antitumor — Apoptosis — Leukemia — DNA fragmentation

We have reported a variety of differentiation-inducing agents for human leukemia cells, including camptothecin,[1] VP16,[2] bufalin,[3,4] geranylgeranylacetone,[5] and daidzein.[6] Some differentiation-inducing agents such as camptothecin,[7–10] VP16[7,8,11] cisplatin,[7,11,12] and all-*trans* retinoic acid[13] can induce apoptosis in tumor and normal cells. We, therefore, examined in the present study whether bufalin, one of the components of bufadienolides in the traditional Chinese medicine *chan'su* prepared from toad venom, can induce apoptosis in human leukemia cells.

MATERIALS AND METHODS

Materials Bufalin was isolated from *chan'su* as described previously.[4] 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO). [methyl-^{3}H]Thymidine, [5-^{3}H]uridine, and L-[4,5-^{3}H]leucine were purchased from the Amersham (Aylesbury, UK). All cancer cells were provided by the Japanese Cell Research Resources Bank.

Cell culture Human cancer cells HL-60, K562, U937, PLC/PRF/5, A431, and HeLa were cultured in the RPMI 1640 medium (Gibco, Glasgow, UK) supplemented with 10% fetal calf serum (FCS). Rat AH66 and PC12 cells and mouse P388 and B16 cancer cells were cultured in MEM medium supplemented with 10% FBS at 37°C.

Separation of peripheral blood cells Human monocytes, polymorphonuclear cells, and lymphocytes were prepared from human heparinized blood of a healthy donor as described previously.[14] Briefly, peripheral blood was diluted with an equal volume of 0.9% NaCl and overlaid onto Histopaque-1077 (Sigma Chemical Co.). After centrifugation at 1,000*g* for 30 min at room temperature, the plasma-medium interface, which contained mononuclear cells, and the medium below the interface, which contained polymorphonuclear cells, were separately collected. The mononuclear cells were primary-cultured and further purified. The purity of the mononuclear cells as determined by peroxidase staining was above 85%. The polymorphonuclear cells were overlaid onto Ficoll-hypaque (Flow Laboratories, Irvine, Scotland). After centrifugation at 270*g* for 30 min at room temperature, the layer containing polymorphonuclear cells was collected. The purity of the polymorphonuclear cells thus collected was above 95% as judged by morphological examination after staining with May-Grunwald-Giemsa.

Cell viability Cell viability was determined by the trypan blue exclusion test.

MTT reduction assay The assay was conducted as described previously.[15] Briefly, 0.2 ml of cell suspension containing 10^4 cells was added to each well of a 96-well

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plate and the cells were immediately treated with the drugs. In the case of cells growing in monolayers, the drugs were added 24 h after seeding of the cells. After culture for 3 days at 37°C, MTT solution (2 mg/ml) 50 μl/well was added and the culture was continued for an additional 4 h. The cells were collected by centrifugation at 120g for 5 min, and the supernatant was discarded. In the case of cells growing in monolayers, the medium was removed by aspiration. The cells were dissolved in dimethyl sulfoxide (200 μl/well) for 10 min, and the absorbance at 540 nm was measured in a 96-well plate. The inhibition was evaluated by comparison with untreated cells.

Morphological estimation of apoptotic cells The apoptotic cells treated with bufalin were fixed, stained with Wright-Giemsa and examined in an oil-immersion microscope. Apoptotic cells were identified as those having condensed and fragmented nuclei, and counted.

Analysis of cell cycle progression The cells treated with bufalin were harvested by centrifugation and washed with phosphate-buffered saline. The cells were fixed with ice-cold 70% ethanol at a cell density of 1×10^6/ml, and then treated with 1 mg/ml RNAse for 30 min at 37°C. Propidium iodide was added to the solution at a final concentration of 50 μg/ml. The cell cycles were analyzed by quantitation of DNA content by flow cytometry according to the method of Noguchi and Browne.[16]

Analysis of DNA fragmentation by agarose gel electrophoresis Cellular DNA was extracted by a reported method.[17] Electrophoresis was performed in 1% agarose gel in 40 mM Tris-acetate buffer (pH 7.4), at 50 V. After electrophoresis, DNA was visualized by ethidium bromide staining.

DNA, RNA and protein syntheses DNA, RNA and protein syntheses were determined after treatment of HL-60 cells or HeLa cells with bufalin by adding ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-leucine, respectively, at a final concentration of 1 μCi/ml, followed by incubation for 1 h. The cells were sedimented with 25% trichloroacetic acid (TCA). In the case of HeLa cells, the cells were scraped off with a policeman, followed by addition of TCA. The precipitate was collected on a glass microfiber filter, and washed 3 times with 2 ml of 5% TCA and twice with 2 ml of ethanol. After drying, the filters were transferred to scintillation vials and radioactivity was counted.

Assay for topoisomerase II activity Topoisomerase II activity of HL-60 cells treated with bufalin was measured as described by Tricoli *et al.*[18] Briefly, HL-60 cells treated with bufalin were washed with phosphate-buffered saline and suspended at a density of 10^8 cells/ml in 5 mM potassium phosphate buffer, pH 7.5, containing 1 mM $MgCl_2$, 1 mM DTT, 0.05% Triton X-100, and 1 mM PMSF. After swelling for 20 min, the cells were lysed by adding an equal volume of 2 M NaCl solution containing 0.1 M Tris-HCl, pH 7.5, 1 mM DTT, 0.05% Triton X-100, and 1 mM PMSF. DNA in the cell lysate was then precipitated by adding one-half volume of 18% polyethylene glycol solution and allowing the mixture to stand for 30 min. After centrifugation at 10,000g for 15 min, the supernatant was collected and protein content was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Topoisomerase II activity was measured using the TopoGEN assay kit (TopoGEN Inc., Columbus, OH). The substrate DNA used was a supercoiled plasmid pRYG, a PUC19 derivative in which the *Sph*I-*Nar*I fragment of PUC19 is replaced with a *Sph*I-*Acc*I fragment containing an alternating repeat of purine-pyrimidine sequence that constitutes a strong eukaryotic topoisomerase PQ-6 cleavage site.

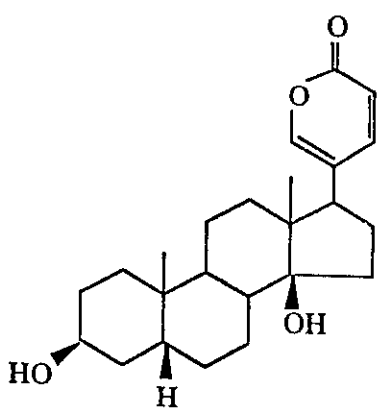

Fig. 1. Chemical structure of bufalin.

Table I. Growth-inhibitory Effect of Bufalin *in vitro* Measured in Terms of MTT Reduction

Tumor cells	IC_{50} (M)
Human	
HL-60 (leukemia)	2.5×10^{-9}
ML1 (leukemia)	2.0×10^{-9}
U937 (leukemia)	3.2×10^{-9}
K562 (leukemia)	5.0×10^{-8}
HeLa (epithelioid carcinoma)	1.0×10^{-7}
PLC/PRF/5 (hepatoma)	2.0×10^{-8}
A431 (epidermoid carcinoma)	1.0×10^{-8}
Mouse	
M1 (leukemia)	$>10^{-5}$
B16 (melanoma)	$>10^{-5}$
P388 (lymphoid neoplasm)	$>10^{-5}$
Rat	
AH66 (hepatoma)	$>10^{-5}$
PC12 (chromaffin cell)	$>10^{-5}$

Cells were treated for 3 days.

RESULTS

The inhibition of growth of various cancer cells by bufalin (Fig. 1), determined in terms of MTT reduction, is shown in Table I. From this table, it can be seen that bufalin selectively inhibits the growth of human cancer cells. The 50% growth-inhibitory concentration (IC_{50}) was in the range of 10^{-9}–10^{-7} *M* for human tumor cells, but was more than 10^{-5} *M* for mouse and rat tumor cells and rat PC12 cells.

We have previously shown that bufalin induces the differentiation of human leukemia cells such as HL-60, ML1, and U937 cells at a concentration of 10^{-9}–10^{-8} *M*.[4] Higher concentrations of bufalin caused the death of these leukemia cells. Fig. 2 shows the effect of bufalin on the viability of HL-60, ML1, and U937 leukemia cells examined by the trypan blue exclusion method. As is evident from this figure, the cell killing by bufalin was dose-dependent. There was no significant difference in the dose-dependent killing effect of bufalin on the three kinds of leukemia cells, ML1, HL-60 and U937.

To investigate whether the cell death caused by bufalin in these leukemia cells is due to the induction of apoptosis, HL-60, U937 and ML1 cells were treated with different concentrations of bufalin for 24 h. As shown in Fig. 3, the cells treated with bufalin exhibited morphological changes. Apoptotic cells were identified by the presence of condensed and fragmented nuclei. The effect

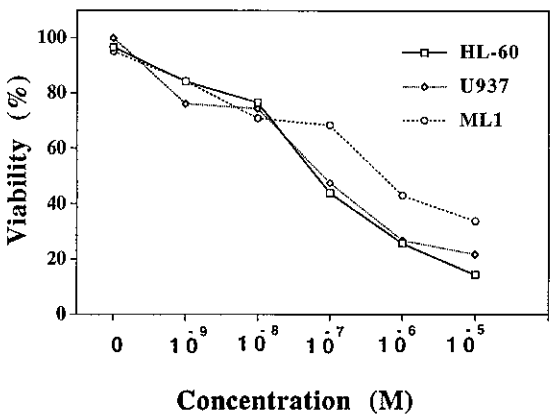

Fig. 2. Effect of bufalin on cell viability. Bufalin was added to exponentially growing cultured leukemia HL-60, U937, and ML1 cells. After 24 h, aliquots of drug-treated and untreated control cells were taken and stained with trypan blue. The percent of viable cells was determined by counting with a hemacytometer.

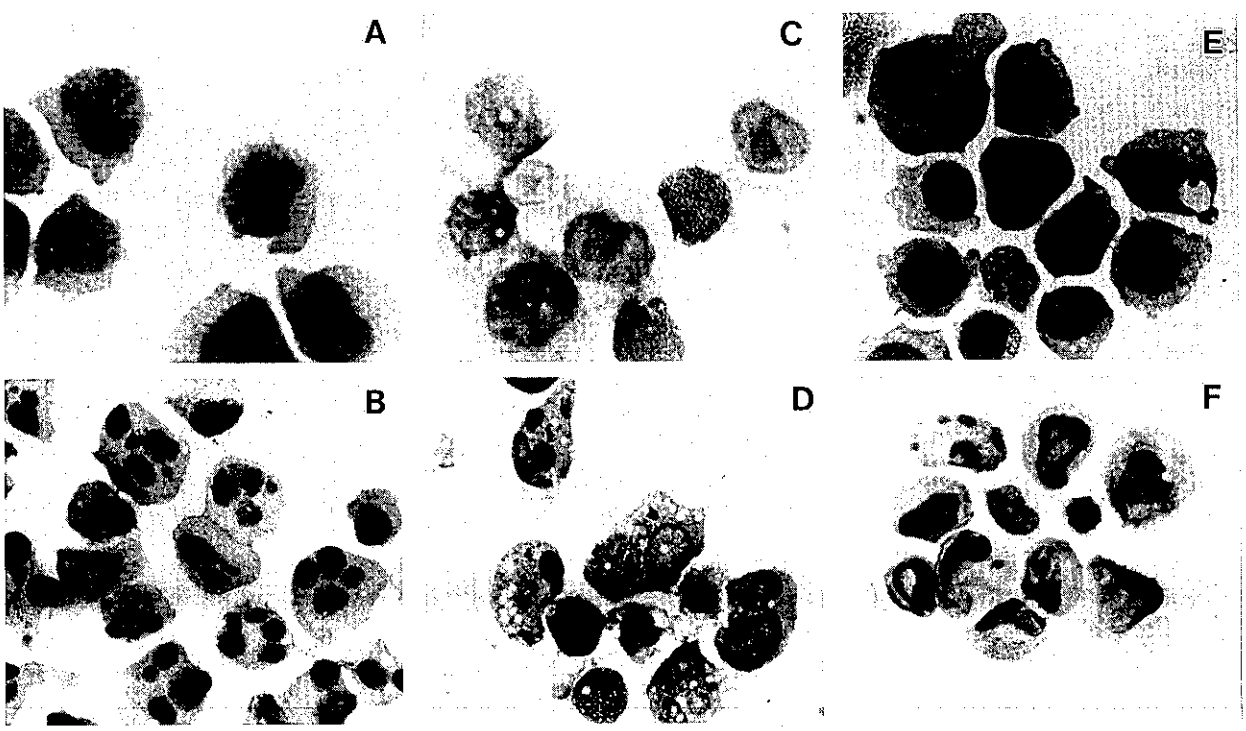

Fig. 3. Morphological appearance of ML1, U937, HL-60 cells treated with 10^{-5} *M* bufalin for 24 h and then stained with Wright-Giemsa. A, C, E: untreated ML1, U937 and HL-60 cells, respectively; B, D, F: 10^{-5} *M* bufalin-treated ML1, U937 and HL-60 cells, respectively.

of bufalin on apoptosis was dose-dependent, and apoptosis was induced in 80% of the cells at the concentration of 10^{-5} *M*.

The effect of bufalin treatment for 24 h on the cell cycle of HL-60, U937 and ML1 cells is illustrated in Fig. 4. Treatment of ML1 cells with different concentrations of bufalin for 24 h arrested the cells in the G2/M phase. This effect increased as the concentration of bufalin was increased from 10^{-9} to 10^{-7} *M*, but when the concentration of bufalin reached 10^{-6} *M*, cell cycle arrest decreased. The cell cycle of U937 cells was also arrested in the G2/M phase by bufalin below 10^{-8} *M*. However, treatment of U937 cells with 10^{-7} to 10^{-5} *M* bufalin resulted in a dramatic increase in the proportion of cells with degraded DNA. Most of the degraded DNA appeared at the position below the G1 peak. Bufalin had no effect on the cell cycle of HL-60 cells at concentrations lower than 10^{-8} *M*. When the concentration was increased beyond 10^{-7} *M*, the HL-60 cells were arrested in the G1 phase and a degraded DNA peak appeared.

Fig. 5 shows the effect of 72-h bufalin treatment on the cell cycle of leukemia cells. ML1 cells treated with bufalin above 10^{-8} *M* were arrested in the G2/M phase and a degraded DNA peak appeared. Treatment of HL-60 and U937 cells for 72 h with bufalin below 10^{-9} *M* had no effect on the cell cycle. The degradation of DNA became evident when ML1 and HL-60 cells were treated with 10^{-7} *M* bufalin for 72 h. Most of the DNA of the U937 cells was degraded by 10^{-7} *M* bufalin.

The result of agarose gel electrophoresis of DNA extracted from HL-60 cells, human mononuclear cells, and polymorphonuclear cells treated with bufalin is shown in Fig. 6. HL-60 cells treated with bufalin above 10^{-8} *M* for 24 h exhibited a ladder pattern characteristic of apoptosis. The DNA ladder of ML1 and U937 cells treated with bufalin was much less pronounced (results not shown). In contrast to HL-60 cells, treatment of mononuclear cells and polymorphonuclear cells with bufalin at 10^{-6} *M*, a concentration 100 times higher than that used for the induction of apoptosis in HL-60 cells, for 24 h did not cause DNA fragmentation.

Since inhibition of topoisomerase activity has been reported to induce apoptosis in various cells,[7-11] we ex-

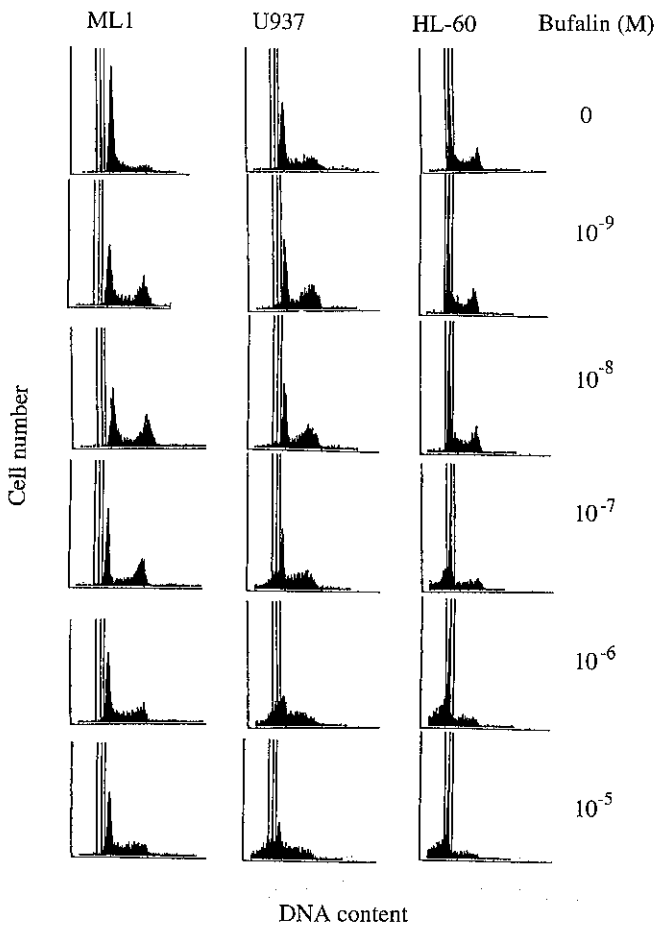

Fig. 4. Cell cycle distribution of HL-60, ML1 and U937 cells treated with various concentrations of bufalin for 24 h. The histograms were generated by flow cytometric analysis of propidium iodide-stained cells.

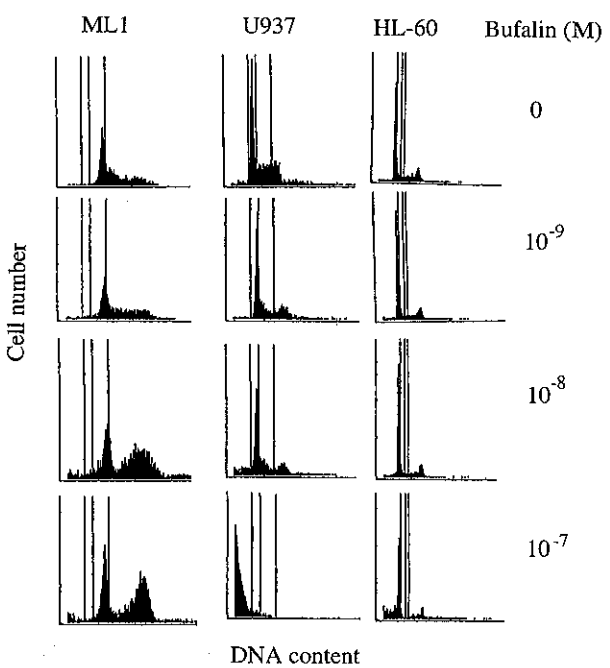

Fig. 5. Cell cycle changes of HL-60, ML1 and U937 cells treated with various concentrations of bufalin for 72 h. The analysis was conducted as described in the legend to Fig. 4.

amined whether bufalin affects the activities of topoisomerases I and II. As shown in Fig. 7, the topoisomerase II activity of HL-60 cells was not changed by 10^{-10} *M* bufalin but was strongly inhibited by bufalin at 10^{-8} *M* and above. Bufalin at 10^{-5} *M* and below did not directly inhibit purified topoisomerase II at all (result not shown). The result indicates that a signal induced in HL-60 cells by bufalin was transmitted to topoisomerase II and resulted in the induction of apoptosis. Topoisomerase I activity of HL-60 cells was not significantly changed by the treatment of the cells with 10^{-8} *M* bufalin (result not shown).

The effect of bufalin on DNA, RNA, and protein syntheses of HL-60 cells was evaluated. The cells were treated with different concentrations of bufalin for 24 h, and the incorporations of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-leucine were measured (Fig. 8). Bufalin showed marked inhibitory effects on DNA synthesis, while the inhibition of RNA and protein syntheses was less marked. For comparison, we examined the effect of bufalin on DNA synthesis of HeLa cells, which are more resistant to bufalin than HL-60 cells, as shown in Table I. In contrast to the marked decrease of DNA synthesis of HL-

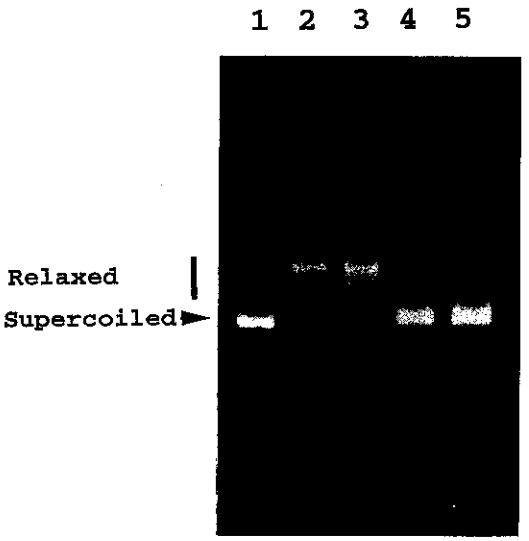

Fig. 7. Topoisomerase II activity in nuclear extracts from HL-60 cells treated with bufalin. HL-60 cells were treated with various concentrations of bufalin for 24 h and the topoisomerase II was extracted as described in "Materials and Methods." The amounts of protein and pRYG used were 5 μg and 125 ng, respectively. HL-60 cells were treated with the following concentrations of bufalin; lane 1, supercoiled pRYG DNA; lane 2, untreated; lane 3, 10^{-10} *M*, lane 4, 10^{-8} *M*; lane 5, 10^{-7} *M*.

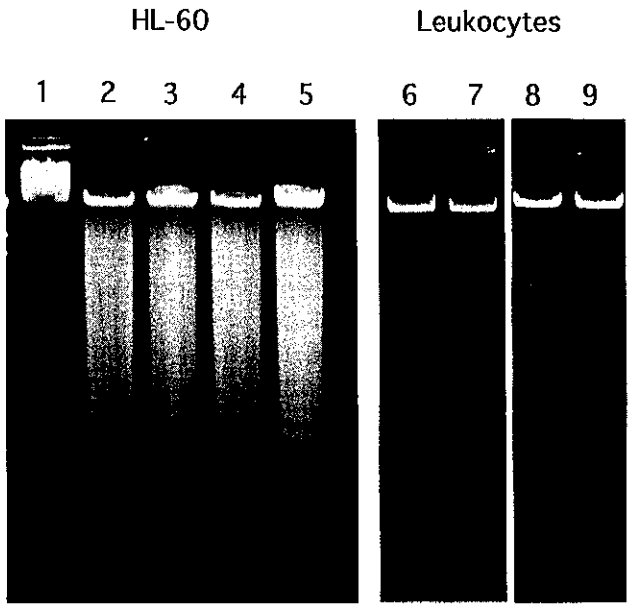

Fig. 6. Agarose gel electrophoresis of DNA extracted from HL-60 cells and human leukocytes treated with bufalin for 24 h. The cellular DNA was extracted as described under "Materials and Methods." Lane 1, DNA from untreated HL-60 cells; cells treated with 10^{-8} *M*, 10^{-7} *M*, 10^{-6} *M*, or 10^{-5} *M* bufalin (lanes 2–5, respectively). Lanes 6 and 8 are untreated mononuclear cells and polymorphonuclear cells; lanes 7 and 9 are mononuclear cells and polymorphonuclear cells treated with 10^{-6} *M* bufalin, respectively.

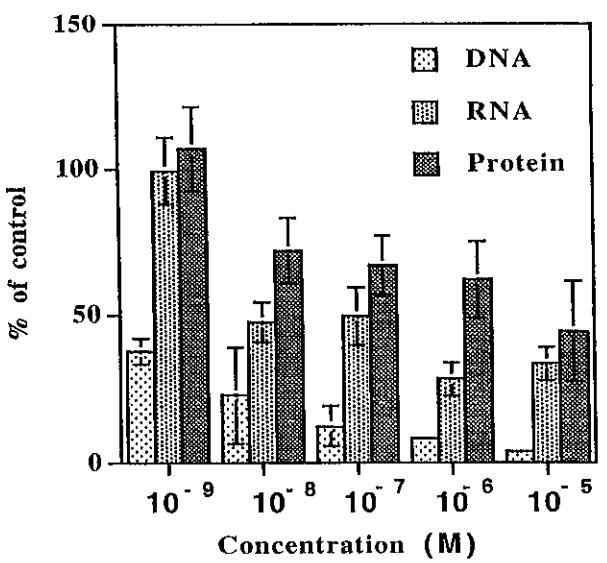

Fig. 8. Effect of bufalin on the DNA, RNA and protein syntheses in HL-60 cells. The cells were treated with various concentrations of bufalin for 24 h. DNA, RNA and protein syntheses were determined by measuring incorporations of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-leucine, respectively, as described in "Materials and Methods." Each value is the mean ± SD of triplicate determinations.

Table II. Comparison of Apoptosis-inducing Activities of Various Antitumor Drugs

Inducer	Concentration (*M*)
All-*trans* retinoic acid	10^{-4}
Cisplatin	4×10^{-6}
Camptothecin	10^{-8}
VP16	2×10^{-6}
Bufalin	10^{-8}

HL-60 cells were incubated with these drugs for 24 h. Above the concentrations indicated, DNA fragmentation of the cells was observed.

60 cells by 10^{-9} *M* bufalin (Fig. 8), DNA synthesis in HeLa cells was not affected at all by 10^{-9} *M* bufalin for 24 h (result not shown). Treatment of HeLa cells with 10^{-8} *M* of bufalin for 24 h decreased DNA synthesis to $66.5\pm5.4\%$ of the control (mean $\pm$ SD for triplicate experiments), but this value is still higher than that observed for HL-60 cells treated with the same concentration of bufalin (Fig. 8). These results suggest that the growth inhibition of human tumor cells by bufalin is mainly due to the inhibition of DNA synthesis.

DISCUSSION

We showed in the present study that bufalin has a potent growth-inhibitory effect on human cancer cells, but it is less potent on mouse and rat tumor cells. Various antitumor drugs such as camptothecin, VP16, cisplatin, and all-*trans* retinoic acid have been reported to induce apoptosis as well as differentiation in many tumor cell types.[7-13] We examined in the present study whether bufalin can also induce apoptosis in human leukemia cells. We demonstrated that bufalin induced typical apoptosis in human leukemia HL-60 cells but not in human leukocytes. HL-60 cells treated with bufalin acquired apoptotic morphological features, extracted DNA showed a ladder pattern when analyzed by gel electrophoresis, and flow cytometry showed fragmented DNA smaller than that of the G1 phase, indicating an association of the antitumor effect of bufalin on HL-60 cells with the induction of apoptosis. ML1 and U937 leukemia cells treated with bufalin also showed degraded DNA on flow cytometry, although these cells did not show a clear DNA ladder pattern when analyzed by gel electrophoresis. Since recent studies have revealed that some cell lines undergo apoptosis without DNA fragmentation,[19,20] further experiments are necessary to determine whether or not the morphological changes induced by bufalin in ML1 and U937 cells are due to apoptosis. It is also necessary to examine whether the growth-inhibitory effect of bufalin on human tumor cells other than HL-60 cells is associated with the induction of apoptosis.

Apoptosis-inducing activities of various antitumor drugs are compared in Table II. As is evident from this table, bufalin seems to be a potent inducer of apoptosis in HL-60 cells. The concentration above which DNA fragmentation of HL-60 cells was observed following treatment with bufalin for 24 h was 10^{-8} *M*, which is much lower than those of all-*trans* retinoic acid, cisplatin, and VP16, and comparable to that of camptothecin.

Apoptosis has been reported to be a mode of cell death of some differentiated cells.[13] Topoisomerase inhibitors were reported to induce terminal differentiation in several cell lines[1,21] and also to induce cell death by either arrest in the G2/M phase or by apoptosis.[21,22] Bufalin, like topoisomerase inhibitors, is able to induce differentiation and typical apoptosis in the human leukemia HL-60 cells, and also can arrest ML1 and U937 cells in the G2/M phase. Topoisomerase II activity of HL-60 cells was indirectly inhibited by bufalin. Therefore, bufalin might bind to certain binding proteins at the cell surface, resulting in transmission of a signal to topoisomerase II to inhibit cell growth and induce apoptosis. A possible candidate of the first target of bufalin in HL-60 cells is Na^+,K^+-ATPase. Cardiotonic steroids are well known to inhibit Na^+,K^+-ATPase activity, and bufalin showed the most potent inhibitory activity on partially purified Na^+,K^+-ATPase from porcine cerebral cortex among cardiotonic steroids such as ouabain, cinobufagin, and digitoxigenin.[4] Although the signal transduction pathway of bufalin from Na^+,K^+-ATPase to topoisomerase II in human tumor cells is unknown at present, it may be different from that in human normal cells and rodent cells. Further work is needed.

(Received December 8, 1993/Accepted March 1, 1994)

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