Jpn. J. Cancer Res. 87, 170–177, February 1996

Cell Death Induced by Baicalein in Human Hepatocellular Carcinoma Cell Lines

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We examined the action of baicalein, a flavonoid contained in the herbal medicine sho-saiko-to (TJ-9), on three cell lines of human hepatocellular carcinoma (HCC). Treatment with baicalein strongly inhibited the activity of topoisomerase II and suppressed the proliferation of all three HCC cell lines. But the mode of cell death induced by baicalein differed according to the cell line. Baicalein induced apoptosis in a concentration-dependent mannner in only one cell line, and an increased concentration of baicalein produced cell death via necrosis in the other two lines. These results suggest that the inhibition of topoisomerase II is not by itself sufficient for induction of apoptosis, and that there is a more important mechanism which can account for the difference in susceptibility of cells to apoptosis induced by baicalein.

Key words: Baicalein — Apoptosis — Hepatocellular carcinoma — Sho-saiko-to (TJ-9) — Cell death

The cancer-preventive and antitumor effects of the herbal medicine sho-saiko-to (TJ-9) on hepatocellular carcinoma (HCC) have been reported by several groups, including ours.[1-5] It has been shown to act as a biological response modifier[6-9] and to induce apoptosis.[10] Which ingredients of TJ-9 may be principally involved in these actions is not known. TJ-9 consists of extracts from seven kinds of herbs. Many of its ingredients are classified as saponins, such as saikosaponin, ginsenoside, and glycyrrhizin, or as flavonoids, such as baicalein, baicalin, and wogonin. We previously showed that saikosaponin, baicalein, baicalin, and glycyrrhizin contained in TJ-9 exert antitumor effects on a human HCC cell line (HuH-7).[11-13] Flavonoids have been reported to inhibit the nuclear enzyme, topoisomerase II,[14] which alters the topological state of DNA through a concerted breaking and rejoining of the DNA strands.[15,16] Some anticancer drugs are known to induce apoptosis via the inhibition of topoisomerase II.[17-20] In this study, we sought to determine whether baicalein (Fig. 1) suppresses cell growth by inducing apoptosis in HCC cells.

MATERIALS AND METHODS

Cell lines Three lines of HCC cells of various degrees of differentiation were used in this study. Well differentiated HCC cells, HuH-7,[21] were maintained with GIT-medium (Wako Pure Chemical Industries, Osaka). Moderately differentiated HCC cells, KIM-1,[22] provided by Dr. M. Kojiro (Kurume University School of Medicine, Kurume) were maintained with Dulbecco's modified Eagle's medium (ICN Biomedicals Japan Co. Ltd., Tokyo) supplemented with 10% fetal bovine serum (Commonwealth Serum Lab., Melbourne, Australia). Poorly differentiated HCC cells, HLF,[23] were maintained with Eagle's minimum essential medium (ICN) supplemented with 20% fetal bovine serum.

Reagents Purified baicalein (Wako) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 100 μg/μl and stored at −20°C. H33258 (Calbiochem-Behring Corp., San Diego, CA) was prepared as a stock solution at a concentration of 1 m*M* in phosphate-buffered saline (PBS) and stored at −20°C in the dark. Anti-Fas (IgM fraction of the mouse monoclonal antibody) was purchased from MBL Co. (Nagoya). Mouse IgM was purchased from Chemicon International, Inc. (Temecula, CA). Fluorescence isothiocyanate (FITC)-conjugated anti-mouse IgM was purchased from Cappel Research Products (Durham, NC). Other general reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Effects of baicalein on cell growth Cells were plated onto 35- ×10-mm style tissue culture dishes (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) and allowed to attach for 24 h. The culture medium was then replaced with media that contained various concentrations of baicalein or a medium that contained only DMSO as a control. Viable cells, as detected by the trypan blue dye exclusion test, were counted every 24 h.

Analysis of morphological changes Cells were cultured in a two-chamber Lab Tek tissue culture slide (Nunc Inc., Naperville, IL), and treated with baicalein (200 μg/ml). At 6, 12, 24, and 48 h after such treatment, the cells were stained with H33258[24,25] (0.01 m*M*) for 1 h, washed with

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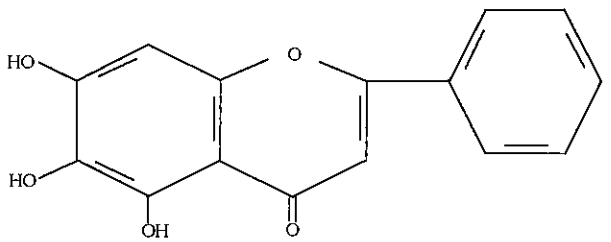

Fig. 1. Chemical structure of baicalein.

PBS, and observed by phase contrast and fluorescence microscopy. Apoptotic cells, identified as those having condensed and fragmented nuclei, were counted, and further studied by electron microscopy.

Analysis of DNA DNA analysis was performed after 48 h of culture with baicalein, using the method of Smith *et al.*[26] The cells were collected by scraping and washed twice with cold PBS. After centrifugation, the pellet was suspended in solution (10^6 cells/500 μl; 10 m*M* Tris-HCl, pH 8.0, 0.1 *M* EDTA, 0.5% sodium dodecyl sulfate), and incubated with RNase (20 μg/ml) for 1 h at 37°C and then with proteinase K (100 μg/ml) for 3 h at 50°C. The suspension was then extracted with phenol/chloroform, and DNA was collected by ethanol precipitation. A 10 μg aliquot of the collected DNA was electrophoresed in a 1.6% agarose gel and visualized by UV illumination after being stained with ethidium bromide, 0.5 μg/ml.

Measurement of DNA fragmentation DNA fragmentation of the cells was assayed as described by Cohen and Duke.[27] Briefly, the cells were lysed with lysis buffer containing 20 m*M* EDTA, 0.5% (v/v) Triton X-100, and 5 m*M* Tris (pH 8.0) for 15 min on ice. The cells were then centrifuged for 20 min at 27,000*g* to separate the intact chromatin (pellet) from the DNA fragments (supernatant). The amount of DNA in the pellet and in the supernatant was measured by using H33258 according to Labarca and Paigen.[25]

Assay for topoisomerase II activity Topoisomerase II activity of HCC cells, whether or not treated with baicalein, was measured with a topoisomerase II assay kit (Topo GEN Inc., Columbus, OH). Topoisomerase II extracts from cells were prepared according to the manufacturer's recommendations. An aliquot of 10^7 cells was treated with baicalein, 200 μg/ml, or with DMSO for 6 h, and harvested by scraping. All extractions were performed at 4°C. Cells were centrifuged (800*g* for 3 min), washed once with 3 ml of ice-cold TEMP buffer (10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 4 m*M* $MgCl_2$, 0.5 m*M* PMSF), and then resuspended in 3 ml of TEMP. After being allowed to swell at 0°C for 10 min, the cells were broken by Dounce homogenization (6–8 strokes). The disruption of cells was monitored by phase-contrast microscopy. Nuclei from the broken cells were collected by centrifugation (1,500*g* for 10 min) and, after resuspension in a small volume (no more than 4 pellet volumes) of TEP (same as TEMP, but lacking $MgCl_2$), an equal volume of 1 *M* NaCl was added. Topoisomerase II was extracted for 1 h on ice with constant stirring. The extract was centrifuged at 15,000*g* for 15 min and the supernatant was assayed. This assay is based on the decatenation of kinetoplast DNA to monomers by topoisomerase II; reaction products are detected by agarose gel electrophoresis.[28, 29]

Expression of Fas antigen Fas antigen is a cell surface protein that is known to trigger apoptosis in a variety of

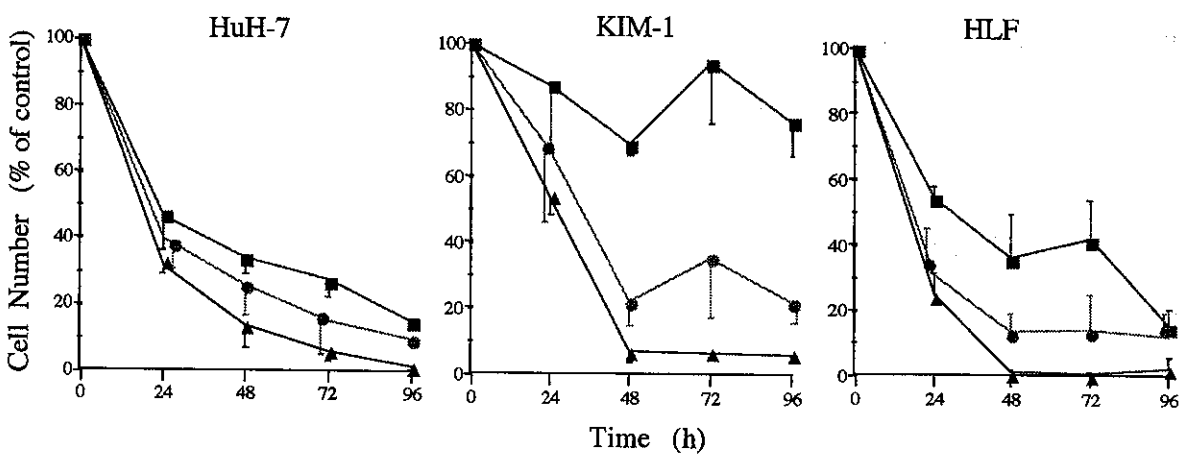

Fig. 2. Effect of baicalein on growth of HCC cells. HCC cells were incubated with baicalein at 50 μg/ml (■), 100 μg/ml (●), or 200 μg/ml (▲). DMSO-treated HCC cells were used as controls. Viable cells were counted by use of the trypan blue dye exclusion test. Data are expressed as percentage of control. Points, mean of triplicate determinations; bars, SD.

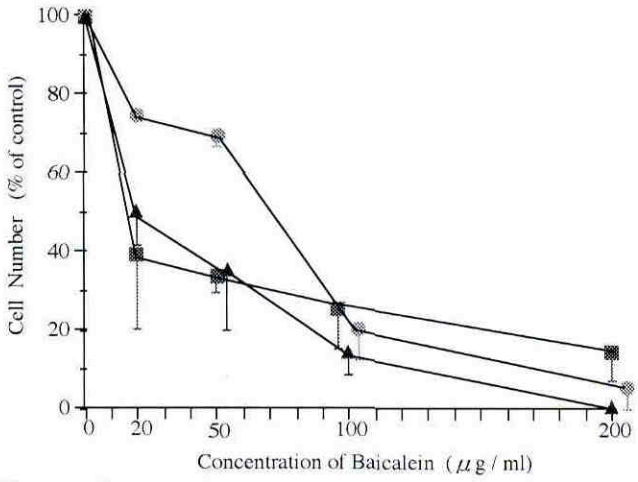

Fig. 3. Concentration-response curves for effect of baicalein on the viability of HCC cells. HuH-7 (■), KIM-1 (●), and HLF (▲) cells were treated with different concentrations of baicalein for 48 h, and the number of viable cells was counted. Data are expressed as the percentage relative to DMSO-treated cells. Points, mean of triplicate determinations; bars, SD.

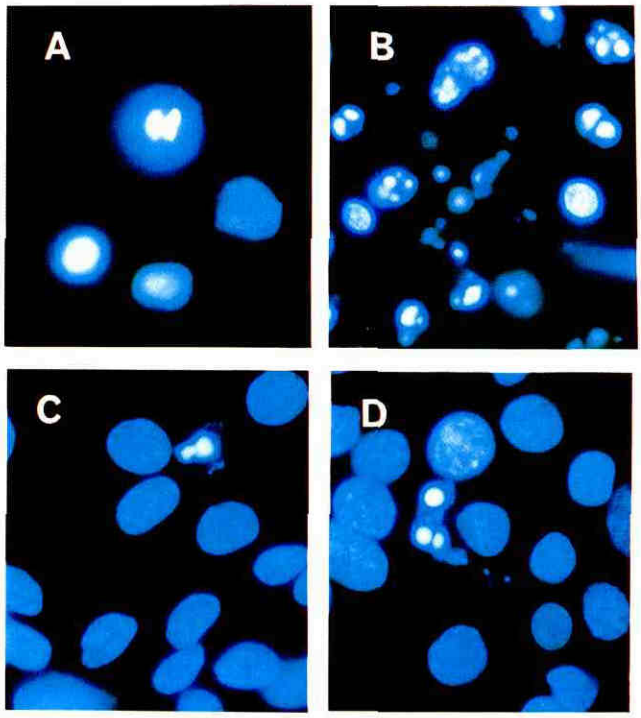

Fig. 4. Morphological changes in HCC cells treated with baicalein, 200 μg/ml (fluorescence microscopic view of H33258 staining, ×200). A, B : KIM-1 cells (A, after 6 h; B, after 48 h of baicalein treatment). C : HuH-7 cells (after 48 h of baicalein treatment). D : HLF cells (after 48 h of baicalein treatment).

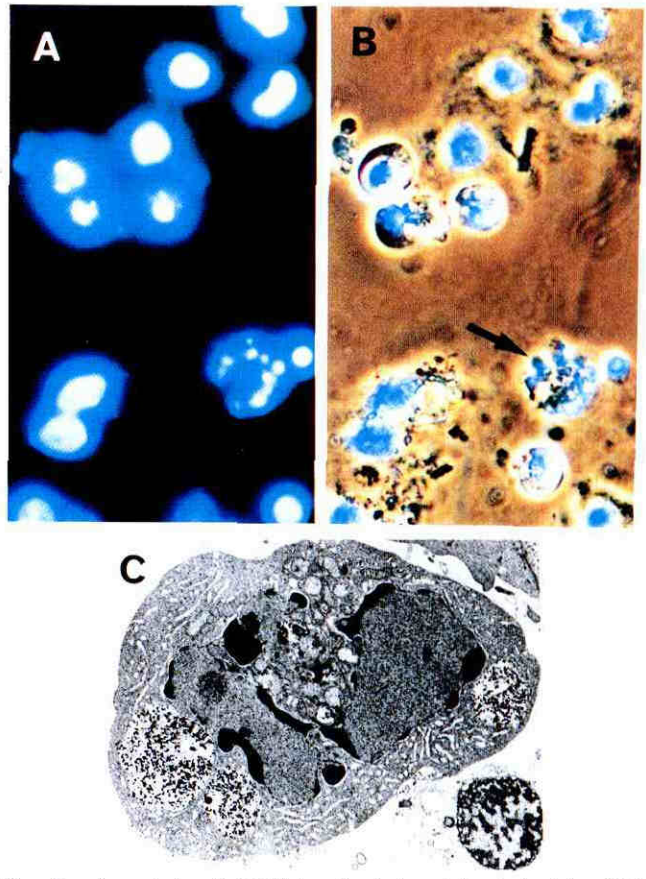

Fig. 5. Apoptosis of KIM-1 cells induced by baicalein, 200 μg/ml. A, B (after 48 h of baicalein treatment) : H33258 staining showing condensed and fragmented nuclei characteristic of apoptosis. Many apoptotic bodies with typical nuclear fragments were formed (arrows). (A, fluorescence microscopic view, ×200 ; B, fluorescence and phase-contrast microscopic view of A, ×200). C: Transmission electron microscopic view showing early nuclear changes in KIM-1 cell treated with baicalein for 12 h. Margination of compacted nuclear chromatin and deeply convoluted nuclear membrane are visible.

cell types upon specific ligand binding.[30-32] To determine whether the susceptibility to apoptosis induced by baicalein was correlated with the expression of Fas antigen, we examined the expression of Fas antigen on HuH-7, KIM-1, and HLF cells by flow-cytometric analysis. Cells that had been trypsinized and washed twice with PBS were incubated for 30 min at 4°C with anti-Fas, 1 μg/ml, or with mouse IgM. After having been washed twice, they were incubated with the secondary antibody, FITC-conjugated anti-mouse IgM, for an additional 30 min. After having been washed twice more, they were subjected to flow cytometric analysis by use of an EPICS profile (Coulter Corp., Hialeah, FL).

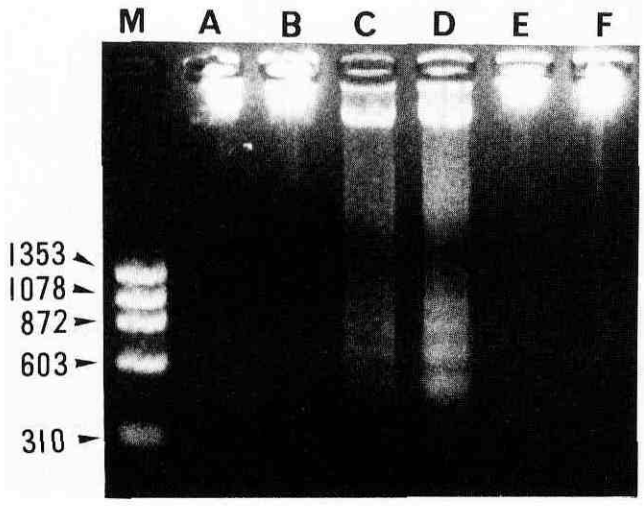

Fig. 6. Agarose gel electrophoresis of DNA extracted from HCC cells treated with baicalein. HCC cells (lanes A–D, KIM-1 cells; lane E, HuH-7 cells; lane F, HLF cells) were treated for 48 h with DMSO alone as a control (lane A) or with baicalein, 50 μg/ml (lane B), 100 μg/ml (lane C), or 200 μg/ml (lanes D–F). Lane M, molecular weight markers. The DNA ladder characteristic of apoptosis was observed in lanes C and D.

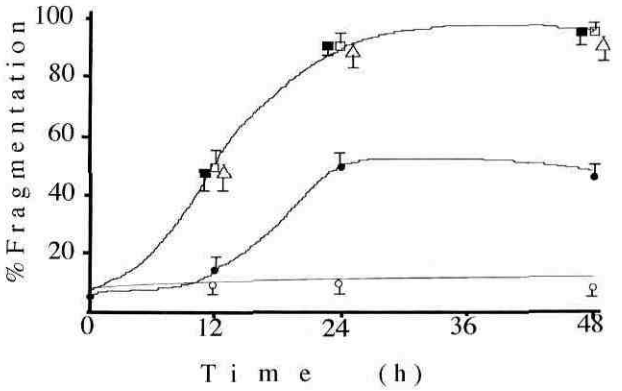

Fig. 7. Time course of DNA fragmentation after treatment with baicalein. Cells were cultured with baicalein, 100 μg/ml (●) or 200 μg/ml (□) or with DMSO alone as a control (○). The effects of concomitant treatment with actinomycin D (■), 100 ng/ml, or with cycloheximide (△), 1.25 μg/ml, with baicalein, 200 μg/ml, were examined. Percentage of DNA fragmentation on the Y-axis is the ratio of fragmented DNA to total DNA. Points, mean of triplicate determinations; bars, SD.

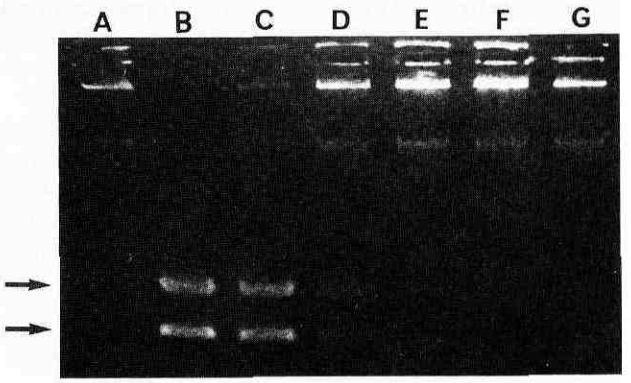

Fig. 8. Topoisomerase II activity in nuclear extracts from HCC cells treated with baicalein. HCC cells (lanes C–E, KIM-1 cells; lane F, HuH-7 cells; lane G, HLF cells) were treated for 6 h with DMSO alone as a control (lane C) or with baicalein, 100 μg/ml (lane D) or 200 μg/ml (lane E–G) and topoisomerase II was extracted as described in "Materials and Methods." Active topoisomerase II decatenates kDNA to monomers (arrows). Lane A, kDNA marker; lane B, topoisomerase II-decatenated kDNA marker.

RESULTS

Effects of baicalein on cell growth Baicalein showed concentration- and time-dependent inhibitory effects on the growth of HuH-7, KIM-1 and HLF cells, as seen in Figs. 2 and 3. The median growth-inhibitory concentration (IC_{50}) was 17 μg/ml for HuH-7, 70.5 μg/ml for KIM-1, and 21 μg/ml for HLF. After 48 h of treatment with baicalein, 200 μg/ml, more than 85% of all three kinds of HCC cells had died.

Effects of baicalein on morphological changes (Figs. 4 and 5) After 6 h of baicalein treatment, apoptotic cells with condensed and fragmented nuclei could be observed (Fig. 3). After 48 h, although apoptosis was induced in 90% of KIM-1 cells, it was evident in less than 10% of HuH-7 and HLF cells.

Electrophoretic analysis of DNA As shown in Fig. 6, a ladder of fragmented DNA of 180 base pairs was observed in KIM-1 cells cultured with baicalein at a concentration of 100 μg/ml or higher. In contrast, no DNA fragmentation was observed in HuH-7 and HLF cells cultured with baicalein (at any concentration), cultures in which there were few morphologically apoptotic cells.

Time course of DNA fragmentation The time course of DNA fragmentation was examined in baicalein-treated KIM-1 cells. As shown in Fig. 7, DNA fragmentation was concentration- and time-dependent; after 48 h, about 90% of the DNA was fragmented in KIM-1 cells treated with baicalein, 200 μg/ml. Actinomycin D or cycloheximide, which can block the *de novo* synthesis of RNA or protein, failed to prevent apoptosis of baicalein-treated cells. Concomitant treatment of cells with baicalein and either actinomycin D (100 ng/ml) or cycloheximide

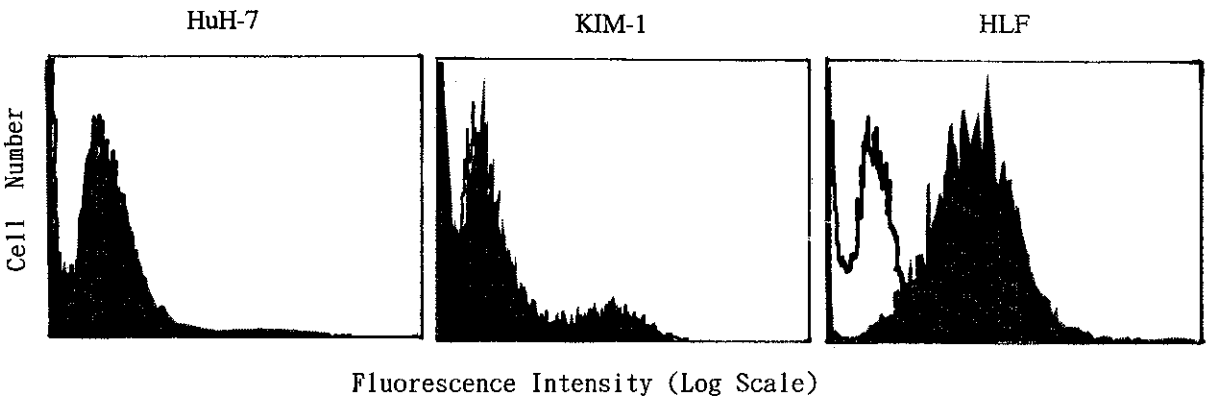

Fig. 9. Expression of Fas antigen on HCC cells, as measured by flow cytometry. Cultured cells were stained with mouse IgM as a control (open area) or with anti-Fas (filled area), as described in "Materials and Methods." The mean fluorescence intensity (MFI) of cells stained with anti-Fas was 3.7 (control 3.5) for HuH-7, 5.7 (control 4.8) for KIM-1, and 15.3 (control 2.7) for HLF.

(1.25 μg/ml) did not alter the percentage of DNA fragmentation (higher concentrations of actinomycin D or cycloheximide caused cell death; data not shown).

Effect of baicalein treatment on extractable topoisomerase II activity As shown in Fig. 8, topoisomerase II activity of HCC cells was strongly inhibited by baicalein (200 μg/ml); decatenated DNA (monomers) was not detected in lanes E–G.

Expression of Fas antigen Of all three HCC cell lines, only HLF was Fas-positive; KIM-1 cells, which were sensitive to baicalein-induced apoptosis were Fas-negative (Fig. 9). Treatment with baicalein did not increase the expression of Fas antigen on the cells (data not shown).

DISCUSSION

The present study showed that baicalein exerted a concentration-dependent, growth-inhibitory effect on HCC cells. Baicalein administered for 6 h inhibited the activity of topoisomerase II in all three cell lines. This observation indicates that the intracellular target of baicalein is topoisomerase II, as has been reported for anticancer agents such as camptothecin and VP-16. The mode of cell death induced by baicalein differed according to the cell line, with apoptosis induced in KIM-1 cells, and necrosis in HuH-7 and HLF cells.

Apoptosis, first described by Kerr *et al.*,[33] occurs via the activation of a cell-intrinsic suicide program, and is morphologically distinct from necrosis. Apoptotic cell death is characterized by cell shrinkage, nuclear condensation and degradation of DNA to oligonucleosomal fragments. It plays a major role as a physiological control mechanism during development and homeostasis.[34] This type of cell death is also involved in pathological conditions[35] such as cancer, with an imbalance between apoptosis and mitosis considered to be a major factor in carcinogenesis.[36–38] Apoptosis has received attention from the viewpoint of cancer therapy, since such artificial triggers as chemotherapeutic agents and anti-Fas antibody induce apoptosis in various tumor cell lines.[39, 40] We and other investigators have reported that TJ-9 inhibits chemical hepatocarcinogenesis in rats.[1, 2] It was recently reported that TJ-9 induces apoptosis in cell lines of hepatobiliary tumors.[10]

Baicalein-treated KIM-1 cells displayed the characteristic morphological features of apoptosis, as revealed by H33258 staining and electron microscopy. DNA analysis of the baicalein-treated cells revealed the characteristic 180-bp ladder. Thus, baicalein induced apoptosis in KIM-1 cells. The incidence of apoptosis in KIM-1 cells depended on the concentration of baicalein. Such apoptosis was not blocked by actinomycin D, an inhibitor of RNA synthesis, or by cycloheximide, an inhibitor of protein synthesis. These findings indicate that baicalein induces apoptosis via a pathway that does not actively involve the *de novo* synthesis of RNA or protein. Although apoptotic cell death is considered to require the expression of new genes, some investigators have reported apoptosis to be independent of the *de novo* synthesis of RNA or protein.[17, 41] In such cases, it is assumed that the machinery that causes apoptosis is already present in the cells.

Death of two cell lines, HuH-7 and HLF, induced by baicalein occurred mainly via necrosis. The question arises, why should the mode of cell death induced by baicalein vary with cell line? Cells can resist apoptosis due to a lack of the gene that activates the apoptotic

process, such as wild-type *p53*,[42,43] or may possess a gene such as *bcl*-2,[44] which may block the process of apoptosis. We detected *p53* mutations in HuH-7 and HLF cell lines[45] with a low percentage of apoptotic cells after baicalein treatment. HuH-7 cells had a mutation of one allele in exon 6 and a deletion of another allele. HLF cells showed both a mutation and a deletion in exon 7. KIM-1 cells exhibited no genetic alterations in exon 5, 6, 7 or 8 of the *p53* gene, which we analyzed. If the apoptosis induced by baicalein occurs via a pathway that requires wild-type p53 protein, the incidence of apoptotic cell death should differ among the three HCC cell lines. Cycloheximide has been reported to block the apoptosis dependent on p53 protein.[18] However, our findings are inconsistent with that finding. The best way of resolving the questions of whether the apoptosis induced by baicalein depends on wild-type p53 protein, and whether this is the reason for the difference in the frequency of apoptosis between cell lines, would be to introduce the wild-type *p53* gene into HuH-7 or HLF cells. It is uncertain whether the wild-type p53 protein would produce a difference in the susceptibility to apoptosis after baicalein treatment. The combined treatment of HuH-7 or HLF cells with baicalein and actinomycin D or cycloheximide did not increase the frequency of apoptotic cell death (data not shown), which suggests that genes such as *bcl*-2 may not suppress the apoptosis induced by baicalein in HuH-7 and HLF cells. It was previously reported that, as the level of the apoptosis-inducing stimulus increases, a switch from apoptosis to necrosis occurs, because the damage to the cells may be too severe to allow activation of an internally programmed mechanism of suicide, and it was further found that the maximum level of stimulus that induces apoptosis differs with the cell lines, reflecting their differing abilities to cope with the stimulus.[46,47] That the median growth-inhibitory concentrations (IC_{50}) of HuH-7 and HLF cells were lower than that of KIM-1 cells raises the possibility that the concentration of baicalein in HuH-7 and HLF cells was high enough to alter the mode of cell death from apoptosis to necrosis. Simply increasing the concentration of baicalein did not induce apoptosis in HuH-7 and HLF cells.

We have shown that Fas-negative KIM-1 cells were sensitive to the apoptosis induced by baicalein, and that baicalein did not induce apoptosis via Fas antigen. Recently, synergy between anti-Fas antibody and chemotherapeutic drugs such as adriamycin or cisplatin has been reported.[40] This raises the possibility that chemotherapeutic drugs may share some mechanism with anti-Fas antibody-mediated signaling events for cytotoxicity. Combination treatment with anti-Fas antibody and baicalein may be effective for induction of apoptosis in Fas-positive cell lines such as HLF cells. Further study is needed.

In conclusion, baicalein induced apoptosis in a concentration-dependent manner in only one of three lines of HCC cells. An increased concentration of baicalein produced cell death via necrosis in the other two lines of HCC cells.

ACKNOWLEDGMENTS

We thank Dr. M. Takahashi (Department of Clinical Laboratory Science, Yamaguchi University School of Medicine) for the electron photomicrograph of baicalein-treated cells.

(Received August 17, 1995/Accepted November 22, 1995)

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