

## The Chinese Herbal Medicine *Tien-Hsien* Liquid Inhibits Cell Growth and Induces Apoptosis in a Wide Variety of Human Cancer Cells

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### ABSTRACT

**Objective:** *Tien-Hsien* liquid (THL) is a commercially available Chinese herbal mixture that has been used as an anticancer dietary supplement for more than 10 years. We recently showed that THL has strong immunomodulatory effects on peripheral blood mononuclear cells (PBMC) and T cells. To investigate the anti-tumor activity of THL further, we sought to test whether THL could induce apoptosis in various human cancer cell lines based on the fact that THL contains several components with tumor killing functions.

**Design:** The growth inhibitory effect of THL on human cervical carcinoma C-33A cells, human lung carcinoma H1299 cells, and human PBMC was assessed by counting viable cells using the trypan blue dye exclusion method. The apoptosis-inducing activity of THL in H1299 cells was assessed by analyzing the cells with four assays: (1) Hoechst 33258 nuclear DNA staining; (2) the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay; (3) the nucleosomal DNA fragment ladder assay; and (4) the sub-G<sub>1</sub> cell analysis. The activities of caspase-8, -9, and -3 in H1299 cells treated with or without THL were also measured to elucidate the possible mechanism underlying THL apoptosis-inducing effect. Finally, the apoptotic effect of THL on fifteen human cancer cell lines and normal human cells were analyzed by the TUNEL assay.

**Results:** THL could induce apoptosis in all human cancer cell lines tested but not in normal human cells. THL treatment of H1299 cancer cells resulted in activation of caspase-8, -9, and -3 and the inhibitors of these caspases could partially block THL-induced apoptosis.

**Conclusions:** THL has been used by numerous patients with cancer for many years with no known adverse effect. Our present study showing that THL had a broad-range tumor killing function has provided a molecular basis underlying THL therapeutic activity. Furthermore, because THL had apoptotic effects only on cancer cells but not on normal cells, this selectivity suggests that THL could be a potential cancer therapeutic agent.

### INTRODUCTION

The popularity of complementary and alternative medicine (CAM) is an international phenomenon. The prevalence of CAM use is estimated at 25% among residents of

the United Kingdom, 50% among German and French populations (Fisher and Ward, 1994), and 34% to 42% among residents of the United States (Eisenberg et al., 1998). Among CAM, herbal medicine has become an increasingly common form of alternative therapy in the United States. A

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survey in 1997 estimated that 12.1% of adults in the United States had used an herbal medicine in the previous 12 months (compared to 2.5% in 1990), resulting in out-of-pocket payments of \$5.1 billion (Eisenberg et al., 1998). Among the many reasons cited by the general public for use of herbal medicines is the belief that botanicals will provide some measure of benefit over and above traditional allopathic medical approaches. This is particularly true for patients with cancer who feel the limitation of conventional cancer treatment and are desperate for new therapy. A summary of 26 surveys across 13 countries concluded that approximately 31.4% of patients with cancer overall had used CAM, with herbal medicine being the most commonly used CAM among these patients (Ernst and Cassileth, 1998).

Many of the classes of phytochemicals in herbal medicine have been proven to have therapeutic effect. In particular, patients with cancer are reported to benefit from treatment with herbal medicine, and survivability in many cases is significantly enhanced (Ho et al., 2002). The anticancer properties of medicinal herbs may be attributed to their antioxidant, immunomodulatory, antimutagenic, cytostatic, and cytotoxic effects (Han 1994; Ho et al., 2002; Huang and Williams, 1999; Wargovich et al., 2001). Many herbal extracts or their components have been reported to be able to induce apoptosis in cancer cells (Galati et al., 2000; Thatte et al., 2000). Among them are commercially available combination of herbs, such as SPES, which contains lyophilized extracts of 15 Chinese herbs (Sadava et al., 2002); PC-SPES, which contains extracts from 8 herbs (Halicka et al., 1997; Sadava et al., 2002); and *Sho-saiko-to*, which consists of crude ingredients extracted from seven herbs (Liu et al., 1998; Yano et al., 1994). Apparently, apoptosis-inducing activity of anticancer herbs plays an important role in their tumor suppressing function.

*Tien-Hsien* liquid (THL, prepared by Feida Union Pharmaceutical Manufactory, El Monte, CA) is a Chinese herbal mixture that has been used as an anticancer dietary supplement for more than 10 years and was recently shown by us to have strong immunomodulatory effects (Sun et al., 2004). It is an aqueous preparation of herbal mixture and consists mainly of extracts from 14 Chinese medicinal herbs: *Cordyceps sinensis* (CS), *Oldenlandia diffusa* (OD), *Indigo pulverata levis* (IPL; also known as *Indigo naturalis*), *Polyporus umbellatus* (PU), *Radix astragali* (RA), *Panax ginseng* (PG), *Solanum nigrum L.* (SNL), *Pogostemon cablin* (PC), *Atractylodes macrocephalae rhizoma* (AMR), *Trichosanthes radix* (TR), *Clematis radix* (CR), *Margarite* (M), *Ligustrum lucidum Ait* (LLA), and *Glycyrrhiza radix* (GR) (Sun et al., 2004). Among these constituent herbs, the following herbs or their components have been shown to have antitumor activity: CS (Bok et al., 1999; Kuo et al., 1994; Nakamura et al., 1999; Yoshida et al., 1989), OD (Sadava et al., 2002; Wong et al., 1996), IPL (Han 1994; Hoessel et al., 1999), PU (Ohsawa et al., 1992; You et al., 1994), RA

(Lau et al., 1994; Kurashige et al., 1999; Cui et al., 2003; Lin et al., 2003), PG (Keum et al., 2000; Kim et al., 2002; Shin et al., 2000), SNL (Hu et al., 1999), TR (Leung et al., 1986; Tsao et al., 1986; Zhang et al., 2001; Zheng et al., 1995), CR (Qiu et al., 1999), LLA (Lau et al., 1994), and GR (Agarwal et al., 1991; Rafi et al., 2002). The antitumor activity of these herbs may be attributed to their antioxidant, antimutagenic, immunomodulatory, cytostatic, or cytotoxic effects (see above references).

Apoptosis, or programmed cell death, is a cell-intrinsic process that is essential for animal development and tissue homeostasis. Malfunction or dysregulation of this tightly controlled mechanism of cell suicide may result in cancer, neurodegenerative diseases, or other pathologic conditions. A variety of extracellular and intracellular signals can trigger an apoptotic response, including cross-linking of so-called death receptors (such as Fas and tumor necrosis factor receptors), ultraviolet and ionizing radiation, anticancer drugs, growth factor deprivation, and overexpression of certain oncogenes and tumor suppressor genes (Chen and Wang, 2002; Gupta, 2003). It is believed that apoptosis contributes to the antitumor activity of most chemotherapeutic drugs (Johnstone et al., 2002; Lowe and Lin, 2000). THL has been used by many patients with cancer with favorable results in over 10 countries. Constituents of THL, including CS (Nakamura et al., 1999), OD (Sadava et al., 2002), PU (Ohsawa et al., 1992; You et al., 1994), PG (Kim et al., 1999a, 1999b; Lee et al., 2000; Liu et al., 2000); SNL (Hu et al., 1999), PC (Park et al., 1998), TR (Tsao et al., 1986; Zhang et al., 2001; Zheng et al., 1995), CR (Qiu et al., 1999), and GR (Rafi et al., 2002), have been shown to have cytotoxic effects on a variety of cancer cells. These results suggest that THL may induce apoptosis in cancer cells. In this study, we tested the apoptosis-inducing ability of THL on 15 human cancer cell lines originating from different organs and normal human cells including human umbilical vein endothelial cells (HUVEC), and human peripheral blood mononuclear cells (PBMC). We found that THL could induce apoptosis in all human cancer cell lines tested but not in normal human cells. THL treatment of cancer cells resulted in activation of caspase-8, -9 and -3, suggesting that THL may induce apoptosis through different apoptotic pathways. Together these data provide a molecular basis underlying antitumor activity of THL.

## MATERIALS AND METHODS

### Cell culture

The human osteosarcoma cell line, U-2OS, the human breast cancer cell lines, BT-474 and MDA-MB-453, the human cervical carcinoma cell lines, C-33A and HeLa, the human hepatocellular carcinoma cell lines, HepG2 and

HuH-7, the human nasopharyngeal carcinoma cell line, NPC-TW04, the human lung carcinoma cell line, H1299, and the human prostate carcinoma cell line, DU145, were routinely grown in Dulbecco's modified Eagle medium (GIBCO BRL Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) in 5% CO<sub>2</sub>. The human B-cell leukemia cell line, BJAB, the human histiocytic lymphoma cell line, U-937, the human T-cell leukemia cell line, Jurkat, the human gastric adenocarcinoma cell line, AGS, and the human prostate adenocarcinoma cell line, PC-3, were cultured in RPMI-1640 medium (GIBCO BRL Life Technologies) supplemented with 10% fetal bovine serum (FBS) in 5% CO<sub>2</sub>. Primary HUVEC were isolated from umbilical cord as described (Jaffe et al., 1973) and maintained in medium 199 (GIBCO BRL Life Technologies) supplemented with 20% FBS, 30 µg/mL endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY), 15 µg/mL heparin (Leo Pharmaceutical Product, Ballerup, Denmark), and 1 mM pyruvate. Human PBMC were isolated from blood samples by Ficoll-Hypaque centrifugation and cultured in RPMI-1640 medium supplemented with 10% FBS. The growth rates of most of the above cancer cell lines and HUVEC are similar (cell doubling times range from 20 to 24 hours), except BT-474, MDA-MB-453, and Jurkat the cell doubling times of which are approximately 48 hours, 36 hours, and 34 hours, respectively. PBMC do not proliferate under our cell culture conditions.

#### *Growth inhibition assay*

Cells were seeded at  $1 \times 10^4$  cells per well in 24-well cell culture plates and incubated for 12 hours. For dose-response assays, cells were fed with fresh media containing various concentrations of THL. Twenty-four hours later, viable cells were counted using the trypan blue dye exclusion method. For time-course experiments, cells were incubated either in fresh media alone or in fresh media containing 1% (v/v) of THL. Viable cells were then counted using the trypan blue dye exclusion method at the indicated times post-treatment.

#### *Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay*

Cells ( $2-5 \times 10^4$  per well) were seeded on the 24-well plate containing each well a 12-mm round cover slide pretreated with poly-L-lysine (0.1 mg/mL; Sigma-Aldrich, Inc., St. Louis, MO), and incubated in appropriate culture media at 37°C. After 12–16 hours incubation, the cells were either treated with 1% (v/v) THL or left untreated. Twenty hours later, the slides containing attached cells were removed from the well and subjected to TUNEL assay as described below. The cells on the slide were fixed in 4% paraformaldehyde

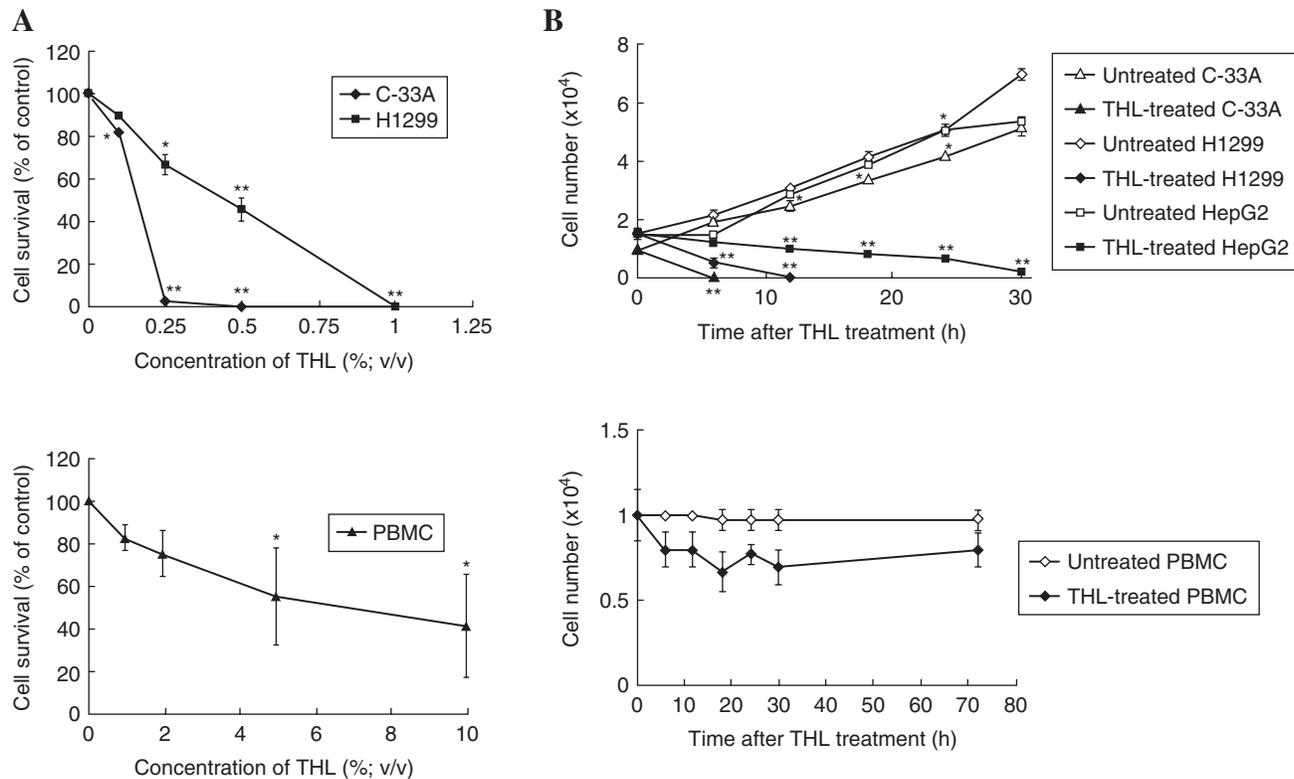
on ice for 15 minutes, washed with phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mmol KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.8 mM KH<sub>2</sub>PO<sub>4</sub>), and permeated with permeabilization solution (0.5% Tween 20, 0.2% bovine serum albumin) at room temperature for 15 minutes. The cells were then labeled with TUNEL reaction mixture, according to the protocol provided by the manufacturer (Roche Applied Science, Mannheim, Germany). Briefly, the permeated cells were rinsed once in PBS, dried, and treated with 15 µL of TUNEL reaction mixture (1.5 µL enzyme solution + 13.5 µL label solution) in a humidified atmosphere at 37°C in the dark for 60 minutes. The enzyme solution contains terminal deoxynucleotidyltransferase from calf thymus, and the label solution is composed of tetramethyl-rhodamine-dUTP and other deoxynucleotides. After reaction, the cells were rinsed three times with PBS containing 0.1% Triton X-100, and incubated in 0.5% propidium iodide (PI; Sigma-Aldrich, Inc.) on ice for 10 minutes to label nuclear DNA. The slide containing labeled cells was then sealed with mounting medium (90% glycerol and 10% PBS) and subjected to fluorescence microscopy examination.

#### *DNA fragmentation assay*

A total of  $2 \times 10^6$  cells were seeded on the 100-mm plate and incubated in appropriate culture media at 37°C for 12–16 hours. The cells were then either treated with 1% (v/v) THL or left untreated for 24 hours. Cellular DNA was extracted using Wizard genomic DNA purification kit, according to the protocol provided by the manufacturer (Promega Corporation, Madison, WI). Briefly, cells were harvested, washed in PBS, and lysed in 600 µL of nuclei lysis buffer. The nuclear lysate was treated with 3 µL of RNase A solution at 37°C for 30 minutes and then incubated with 200 µL of protein precipitation solution on ice for 5 minutes. The lysate was centrifuged at 15,000g at 4°C to remove proteins from the lysate. The clear supernatant containing cellular DNA was then precipitated with 600 µL of isopropanol, washed with 70% ethanol, air dried, and dissolved in DNA rehydration solution. To analyze fragmented DNA, 20 µg of cellular DNA extracted as described above was electrophoresed through a 2% agarose gel, and DNA in the gels was visualized under UV light after staining with 5 µg/mL of ethidium bromide solution.

#### *Apoptosis assay by flow cytometry*

Cells were treated with or without 1% (v/v) THL. After treatment, cells were harvested, fixed with 75% ethanol for 12 hours at 4°C, followed by two washes with PBS. The fixed cells were then incubated in PBS containing PI at 20 µg/mL and RNase A (Sigma-Aldrich, Inc.) at 1 mg/mL at 37°C for 30 minutes. DNA content was determined by flow cytometry on FacScan (Becton Dickinson, San Jose, CA).



**FIG. 1.** Inhibition of cancer cell growth by THL. **A.** Dose-dependent effect of THL on C-33A and H1299 cancer cells (upper panel) and normal human PBMC (lower panel). Cells were cultured in media containing various concentrations of THL for 24 hours. Viable cells were counted using the trypan blue dye exclusion method and expressed as a percentage of the control (without addition of THL). The data represent the average of three independent experiments with the standard deviation. \* $P < 0.05$  and \*\* $P < 0.001$  using an unpaired Student's  $t$ -test for the comparison between untreated and THL-treated cells. **B.** Time-dependent growth-inhibitory effect of THL on C-33A, H1299, and HepG2 cancer cells (upper panel) and normal human PBMC (lower panel). Cells were cultured in media in the absence or presence of 1% (v/v) THL for 6, 12, 18, 24, 30, and 72 hours. Viable cells were counted using the trypan blue dye exclusion method. The data represent the average of three independent experiments with the standard deviation. \* $P < 0.05$  and \*\* $P < 0.001$  using an unpaired Student's  $t$ -test for the comparison between untreated and THL-treated groups at the specific time point.

The FL2 intensity was plotted as histograms on a liner scale. Apoptotic cells were shown as a subdiploid peak.

#### Determination of caspase-3, -8, and -9 activities

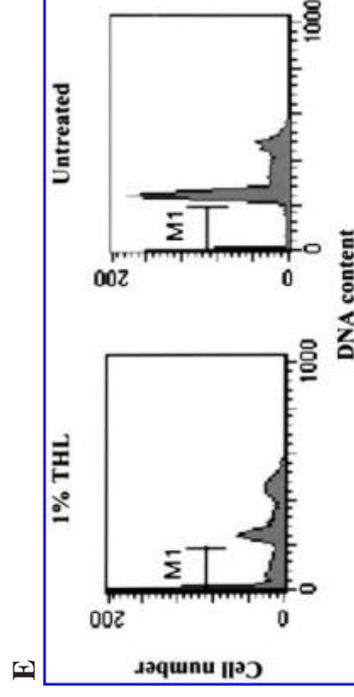
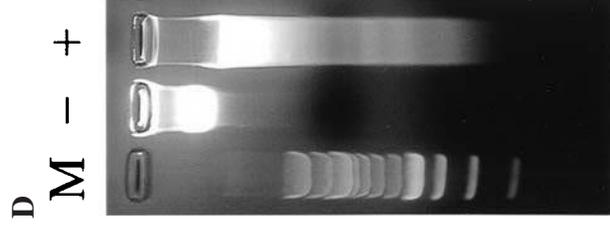
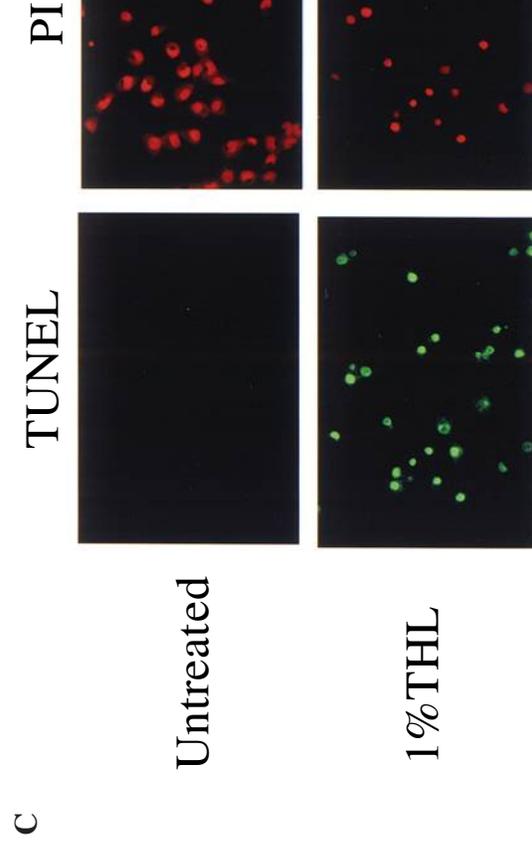
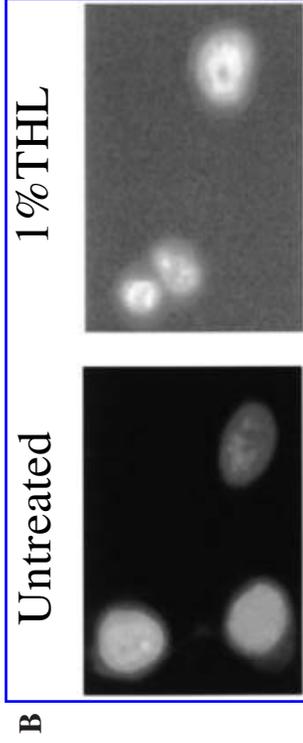
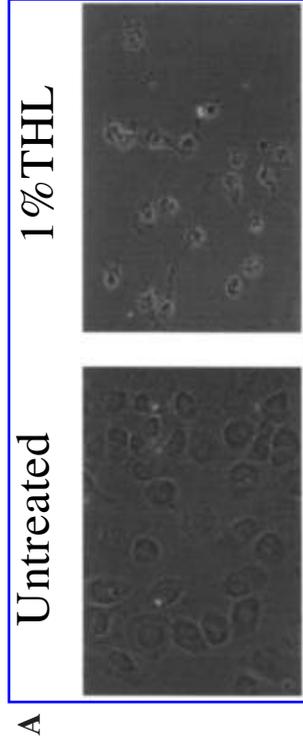
Caspase-3, -8, and -9 activities were assayed by using AFC-conjugated substrates supplied by Calbiochem, Darmstadt, Germany. H1299 cells were treated with 1% (v/v) THL for various lengths of time, harvested, washed with PBS, resuspended in 50  $\mu$ L of chilled cell lysis buffer (1% Triton X-100, 1% Nonidet P-40) containing 1 $\times$  protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany), and incubated on ice for 10 minutes. Cell lysates were cleared by centrifugation and the protein concentration of the supernatants were determined. Aliquots of the supernatants (corresponding to 20  $\mu$ g of total protein) were assayed for caspase activity in 2 $\times$  reaction buffer (10 mM HEPES pH 7.4, 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10 mM dithiothre-

itol) containing 50  $\mu$ M of the fluorogenic caspase-specific substrate (Z-DEVD-AFC for caspase-3, Z-IETD-AFC for caspase-8, and Ac-LEHD-AFC for caspase-9). The caspase reaction was allowed to proceed for 1 hour at 37°C. Under these conditions, caspase activity is proportional to the release of AFC from the substrate. Fluorescence from AFC was monitored with the fluorometer Spectra Max Gemini XS (Molecular Devices, Sunnyvale, CA) using 400 nm excitation and 505 nm emission wavelengths.

## RESULTS

#### Effect of THL on cell growth

The effect of THL on the growth of human cervical carcinoma C-33A cells, human lung carcinoma H1299 cells, and human PBMC was assessed by counting the number of viable cells surviving after THL treatment for 24 hours. As



**FIG. 2.** THL induced apoptosis in human lung carcinoma H1299 cells. **A.** Morphologic changes of the H1299 cells treated with THL. H1299 cells treated for 12 hours without (left) or with (right) 1% (v/v) THL were photographed under a phase-contrast microscope. **B.** Fluorescent staining of nuclei of H1299 cells treated for 12 hours without (left) or with (right) 1% (v/v) THL by Hoechst 33258. The photographs were taken under a fluorescence microscope. **C.** TUNEL staining of H1299 cells untreated or treated with 1% (v/v) THL. The experiments were performed as described in the Materials and Methods section of the text. The cells were first processed with TUNEL to detect the fragmented nucleosomal DNA and then stained with PI to label the nuclear DNA. The same microscopic field was pictured under a fluorescence microscope using a blue or green filter, respectively. **D.** DNA fragmentation assay of H1299 cells untreated or treated with 1% (v/v) THL. Twelve (12) hours after THL treatment, genomic DNA was extracted as described Materials and Methods, electrophoresed in a 2% agarose gel, and visualized with ethidium bromide staining. M, 100-bp DNA ladder size marker; -, untreated H1299 cells; +, THL-treated H1299 cells. **E.** Histograms of DNA content analysis of H1299 cells untreated (right) or treated (left) with 1% (v/v) THL. Ten (10) hours after THL treatment, cells were harvested and subjected to sub-G1 DNA content analysis. The marked region represents percent of apoptotic cells in the sub-G1 peak.

shown in Figure 1A, THL showed a strong and dose-dependent inhibitory effect on the growth of both C-33A and H1299 cells. The concentration required for 50% inhibition of growth of C-33A and H1299 cells ( $ID_{50}$ ) was 0.13% (v/v) and 0.45% (v/v), respectively. At a concentration of 0.5% (v/v) and 1% (v/v), respectively, all of the C-33A and H1299 cells were killed. In contrast, PBMC was only marginally inhibited by 2% (v/v) THL and more than 40% of PBMC survived after 10% (v/v) THL treatment. When C-33A, H1299, and human hepatocellular carcinoma HepG2, and PBMC were incubated with 1% (v/v) THL for various lengths of time, all three cancer cell lines were killed by THL in a time-dependent manner (Fig. 1B). C-33A, H1299, and HepG2 cells were completely killed by THL at 6, 12, and 30 hours, respectively, post-treatment. In contrast, most of PBMC stayed alive even at 72 hours post-treatment. Taken together, the above data suggest that THL could specifically kill human cancer cells but not normal human cells at low concentration.

#### *THL could induce apoptosis in human lung carcinoma H1299 cells*

Because THL could cause the death of H1299 cells, we thus investigated whether THL could induce apoptosis in these cells. The cells were evaluated for evidence of apoptosis by four assays: (1) a morphologic study using Hoechst 33258 DNA staining; (2) the TUNEL assay; (3) the nucleosomal DNA fragment ladder assay; and (4) the sub- $G_1$  cell analysis.

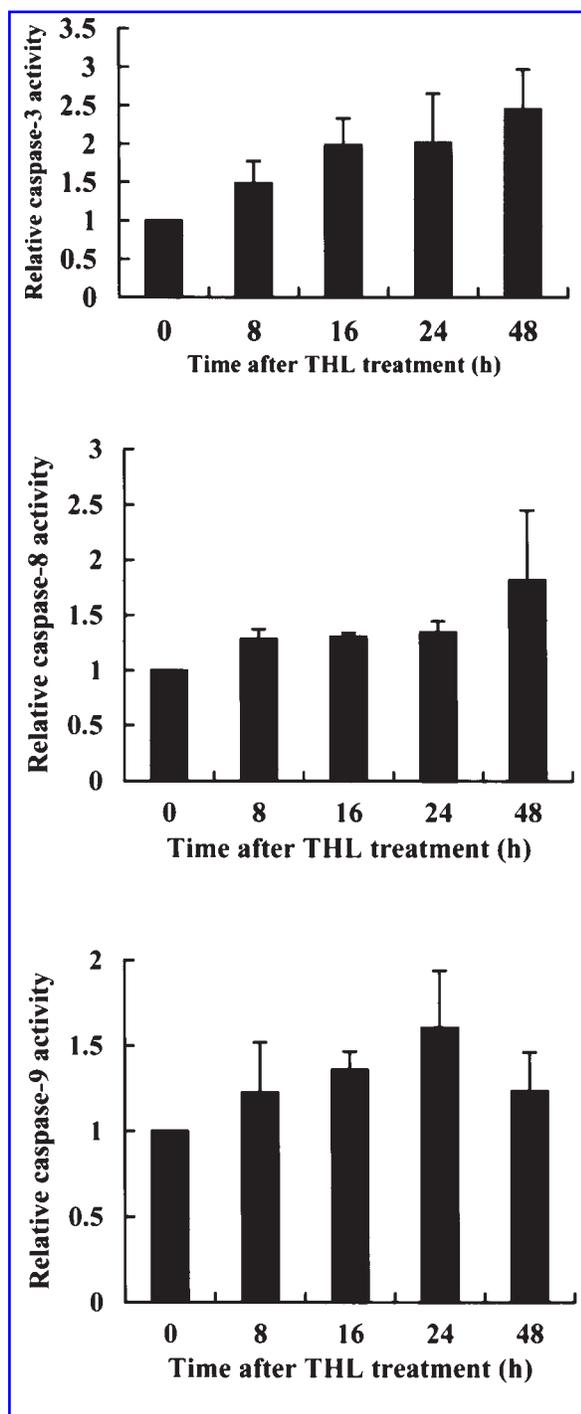
Typical morphologic changes induced by 1% (v/v) THL in H1299 cells are demonstrated in Figure 2A. It was observed that THL treatment caused evident cytoplasmic shrinkage, cell rounding, and membrane blebbing, all characteristic morphologic alterations of apoptosis (Cohen, 1993). When THL-treated H1299 cells were stained with Hoechst 33258, the cells showed typical apoptotic nuclear changes, such as chromatin condensation and nuclear fragmentation (Fig. 2B; Cohen, 1993). We next performed TUNEL staining on 1% (v/v) THL-treated H1299 cells. TUNEL staining quantitates apoptotic chromosome fragmentation by labeling the free 3'-OH of fragmented DNA with terminal deoxynucleotidyltransferase and fluorescein-dUTP (Gavrieli et al., 1992). Compared to untreated control, a majority of the THL-treated cells were TUNEL positive (with fluorescent nuclei), indicating that THL-treated cells were undergoing apoptosis (Fig. 2C). The nucleosomal DNA fragment ladder assay was then used to monitor apoptosis induced by THL. DNA fragmentation in a ladder pattern indicates internucleosomal chromatin cleavage, which is a characteristic of apoptosis (Wyllie, 1980). As shown in Figure 2D, a typical "ladder" of approximately 200-bp multiple DNA fragments oc-

curred in H1299 cells treated with 1% (v/v) THL but not in untreated control cells, further confirming that THL could induce apoptosis in H1299 cells. Finally, flow-cytometric analysis of PI-stained cells was performed with THL-treated and untreated H1299 cells. Cells with subdiploid (sub- $G_1$ ) DNA contents were scored as apoptotic based on the previous study (Nicoletti et al., 1991). As shown in Figure 2E, about 53.8% of THL-treated H1299 cells contained sub- $G_1$  DNA content, in sharp contrast to untreated cells that had only 3.1% of cells in the sub- $G_1$  peak. Taken the above data together, we conclude that THL can induce apoptosis in H1299 cells.

#### *Activation of caspase-8, -9, and -3 by THL*

Many of the cellular changes that occur during apoptosis are a result of the activity of the caspase family of cysteine proteases (Chen and Wang, 2002). Caspases involved in apoptosis are generally divided into two categories, the initiator caspases and the effector caspases. Among the initiator caspases, caspase-8 plays a critical role in the death receptor-mediated apoptosis while caspase-9 is required for the mitochondrial death pathway (Chen and Wang, 2002; Gupta, 2003). Caspase-3, an effector caspase, is a key executioner of apoptosis mediated by various apoptotic stimuli. Both death receptor- and mitochondrion-mediated apoptotic signals activate caspase-3, which in turn proteolytically cleaves many final key apoptosis proteins (Chen and Wang, 2002; Gupta, 2003).

To investigate whether caspase-8, -9, and -3 were involved in the THL-mediated death response, we measured the activities of caspase-8, -9, and -3 in H1299 cells after treatment with THL. The fluorimetric caspase activity assays with Z-IETD-AFC, Ac-LEHD-AFC, and Z-DEVD-AFC as substrates for caspase-8, -9, and -3, respectively, were performed. Data in Figure 3 demonstrate that caspase-8, -9, and -3 were activated in a time-dependent manner upon treatment with THL. The activity of caspase-3 was induced more than twofold at 48 hours post-treatment, while that of caspase-8 and -9 was induced at lower level. To investigate the involvement of caspase-8, -9, and -3 in the THL-mediated apoptosis further, we used inhibitors to block their proteolytic activity in THL-treated H1299 cells. As shown in Figure 4, addition of caspase-8, -9, or -3 inhibitor alone could more or less block THL-induced apoptosis. Combination of caspase-8 and -9 inhibitors was more potent than single inhibitor in blocking THL-induced apoptosis, while combination of caspase-8, -9, and -3 inhibitors was most potent in this apoptosis blocking effect. Taken together, these data indicate that all three caspases play distinct roles in THL-induced apoptosis, and suggests that both death receptor- and mitochondrion-mediated apoptotic pathways are involved in THL-mediated death



**FIG. 3.** Time-dependent activation of caspase-3, -8, and -9 in H1299 cells by THL. H1299 cells were treated with 1% (v/v) THL for the indicated periods. Caspase activities were determined using fluorimetric caspase activity assay as described in the Materials and Methods section of the text. Relative caspase activity was obtained by comparing the enzyme activities acquired after THL treatment with that acquired before THL treatment. The data represent the average of three experiments with the standard deviation.

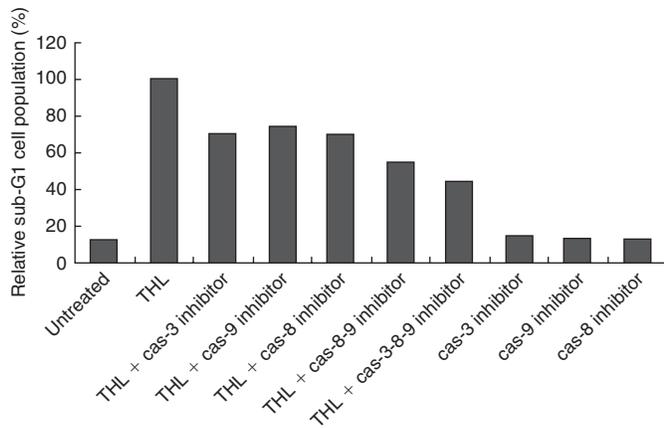
response. Moreover, since combination of these caspase inhibitors could not completely block THL-induced apoptosis (Fig. 4), it is possible that caspase-independent pathways are also involved in THL-mediated apoptotic response.

#### *THL induced apoptosis in a broad spectrum of human cancer cells*

Because THL also caused the death of human cervical carcinoma C-33A cells and human hepatocellular carcinoma HepG2 cells (see Fig. 1), we thus investigated whether THL could induce apoptosis in these cancer cells and other cancer cells. As evident by the formation of internucleosomal DNA fragments, treatment with 1% (v/v) THL induced apoptosis in C-33A, HepG2, human hepatocellular carcinoma HuH-7, and human nasopharyngeal carcinoma NPC-TW04 cells, but not in normal HUVEC (Fig. 5). TUNEL assays also indicated that while the nuclei of untreated C-33A, HepG2, HuH-7, and NPC-TW04 cancer cells were stained TUNEL-negative (data not shown), the nuclei of these cancer cells after 1% (v/v) THL treatment were stained TUNEL-positive (Fig. 6). In contrast, both THL-treated and untreated HUVEC were stained TUNEL-negative (Fig. 6; data of untreated group not shown). Taken together, these data strongly suggest that THL could specifically induce apoptosis in human cancer cells but not in normal human cells.

To investigate the apoptosis-inducing ability of THL further, we performed TUNEL assays on 15 human cancer cell lines and normal HUVEC and PBMC after treatment with 1% (v/v) THL. As shown in Table 1, while cancer cells originating from different human organs all underwent apoptosis (TUNEL-positive), the normal HUVEC and PBMC did not. Although PBMC cannot proliferate under our cell culture conditions (Fig. 1B), HUVEC did proliferate as well as most of the cancer cell lines tested (data not shown). Therefore, THL cannot induce apoptosis in both proliferating and resting normal human cells. Together these data further confirm the specific cancer-killing function of THL and suggest that THL may have therapeutic effect on a broad spectrum of cancers.

It has been shown previously that polyphenolic compounds in herbs may lead to generation of  $H_2O_2$  in commonly used cell culture media (such as DMEM), and the resulting  $H_2O_2$  can cause apoptosis of the cells (Chai et al., 2003). To rule out the possibility that the apoptosis-inducing effect of THL is the result of  $H_2O_2$  generated upon THL addition to the culture medium, we added catalase (1000 units per milliliter) to the culture media to remove  $H_2O_2$  if it was generated. As shown in Figure 7, THL could still efficiently induce apoptosis of HuH-7, C-33A, H1299, HepG2, NPC-TW04, and U-2OS cancer cells



in the presence of catalase. These data clearly indicate that the killing effect of THL in cell cultures is directly the result of THL itself but not to artefacts caused by  $H_2O_2$ .

## DISCUSSION

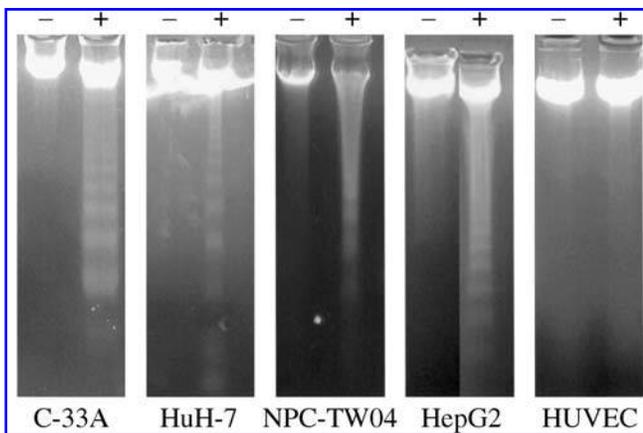
The study presented here demonstrates that THL, a Chinese herbal mixture, induced apoptosis in human lung carcinoma H1299 cells as judged by four criteria: appearance of typical condensed chromatin and fragmented nuclei in cells, positive labeling of cells by TUNEL, DNA fragmentation, and detection of sub- $G_1$  DNA content by flow cytometry. By TUNEL assays, we also showed that THL had strong apoptosis-inducing effects on a broad spectrum of hu-

**FIG. 4.** Effects of caspase inhibitors on THL-induced apoptosis. Bars indicate relative sub- $G_1$  cell populations. The level of apoptosis (as indicated by sub- $G_1$  cell population) induced by 1% (v/v) THL was set to 100%. From left to right: Bar 1, H1299 cells without any treatment; bar 2, H1299 cells treated with 1% (v/v) THL; bars 3, 4, and 5, H1299 cells were preincubated with 40  $\mu$ M of caspase-3 inhibitor Z-DEVD-FMK (Calbiochem, Darmstadt, Germany), caspase-9 inhibitor Z-LEHD-FMK (Enzyme Systems, Livermore, CA, USA), or caspase-8 inhibitor Z-IETD-FMK (Enzyme Systems, Livermore, CA, USA), respectively, for 1 hour before exposure to 1% (v/v) THL; bars 6, and 7, H1299 cells were preincubated with combination of inhibitors of caspase-8 (40  $\mu$ M) and -9 (40  $\mu$ M), or combination of inhibitors of caspase-3 (40  $\mu$ M), -8 (40  $\mu$ M) and -9 (40  $\mu$ M), respectively, for 1 hour before exposure to 1% (v/v) THL; bars 8, 9, and 10, H1299 cells treated with 40  $\mu$ M of caspase-3, -9, or -8 inhibitor, respectively.

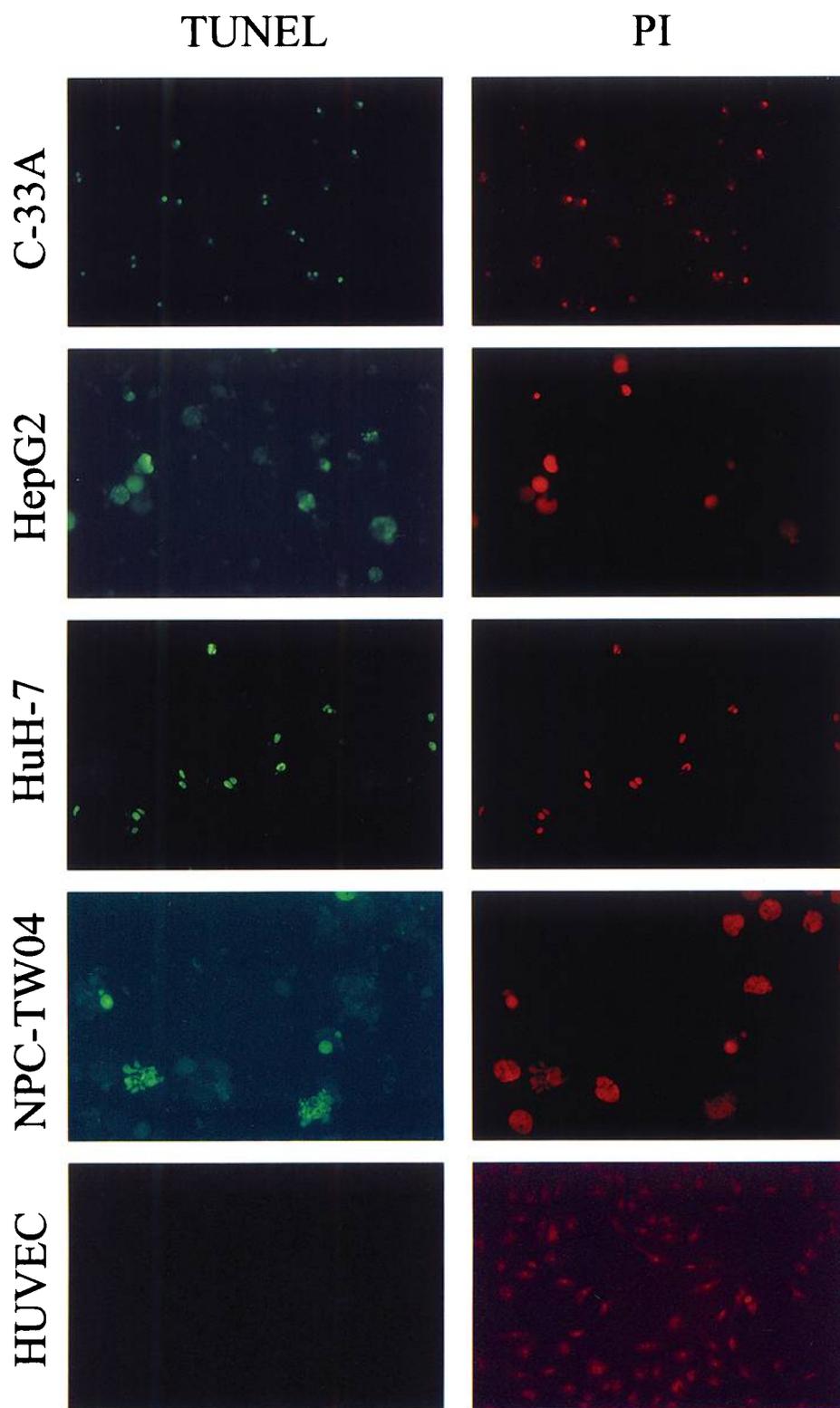
man cancer cell lines but not on normal HUVEC and PBMC (Fig. 2, 5, and 6; Table 1). This selectivity has great therapeutic importance and makes THL a potential cancer therapeutic agent. In this regard, it is worth noting that THL has been used by numerous patients with cancer for many years with no known adverse effect.

THL contains extracts mainly from 14 Chinese medicinal herbs (Sun et al., 2004). Among these constituent herbs, many have been shown to have cytotoxic effects on a variety of cancer cells. Apoptosis is a mechanistically driven form of cell death that is often activated by anti-cancer drugs. Previous studies have shown that several component herbs of THL can induce apoptosis in cancer cells. These include OD, which induces apoptosis in small-cell lung carcinoma cells (Sadava et al., 2002); PG, whose components (ginsenosides) were shown to cause apoptotic death of prostate carcinoma, myeloid leukemia, glioma, and hepatoma cells (Kim et al., 1999a, 1999b; Lee et al., 2000; Liu et al., 2000; Shin et al., 2000); TR, whose component (trichosanthin) induces apoptosis in choriocarcinoma cells by stimulating the production of reactive oxygen species (Zhang et al., 2001); GR, which induces apoptosis in breast and prostate tumor cells (Rafi et al., 2002). In this study, we found that THL had potent apoptotic effects on 15 human cancer cell lines originating from different organs. This broad apoptosis-inducing activity of THL may stem from its complex composition capable of eliciting multiple death signal pathways. These component herbs may act synergistically or additively to induce apoptosis in various cancer cells.

For a tumor cell to propagate, it must survive drastic metabolic stress (e.g., hyperproliferation stimulated by activated oncogenes) and structural alterations (e.g., altered cell adhesion), as well as an extremely stressful microenvironment (e.g., hypoxic conditions and nutrient deprivation). In addition, it must overcome or evade the host anti-tumor immune response. Each of these hurdles may put an



**FIG. 5.** DNA fragmentation assay of various human cancer cells (C-33A, HuH-7, NPC-TW04, and HepG2) and normal human cells (HUVEC) untreated or treated with 1% (v/v) THL. Genomic DNA was extracted as described in the Materials and Methods section of the text, electrophoresed in a 2% agarose gel, and visualized with ethidium bromide staining. -, untreated cells; +, THL-treated cells.



**FIG. 6.** TUNEL staining of various human cancer cells (C-33A, HepG2, HuH-7, and NPC-TW04) and normal human cells (HUVEC) treated with 1% (v/v) THL. The experiments were performed as described in the Materials and Methods section of the text. The cells were first processed with TUNEL to detect the fragmented nucleosomal DNA and then stained with PI to label the nuclear DNA. The same microscopic field was pictured under fluorescence microscope using a blue or green filter, respectively.

TABLE 1. APOPTOTIC EFFECTS OF THL ON VARIOUS CANCER CELLS AND NORMAL HUVEC AND PBMC<sup>a</sup>

Cell	TUNEL <sup>b</sup>
BJAB	++
U-2OS	+++
BT-474	++
MDA-MB-453	++
C-33A	+++
HeLa	++
U-937	++
Jurkat	++
HuH-7	+++
HepG2	++
H1299	+++
NPC-TW04	++
DU145	++
PC-3	+
AGS	+++
HUVEC	-
PBMC	-

<sup>a</sup>Cells were treated with 1% (v/v) THL and subjected to TUNEL staining.

<sup>b</sup>+++ , >80% of cells were TUNEL-positive; ++ , 40–80% of cells were TUNEL-positive; + , about 10% of cells were TUNEL-positive; - , <0.1% of cells were TUNEL-positive.

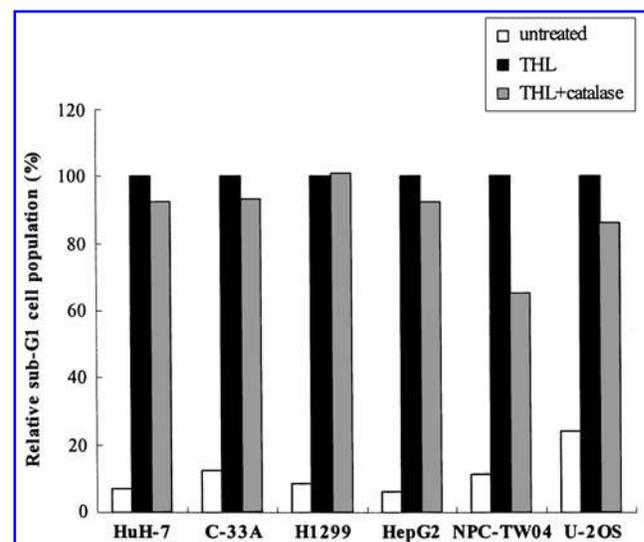
enormous pressure on a cancer cell and cause its apoptotic death. Therefore, for a tumor to grow, it must disable its apoptotic programs. This is the reason why most tumors, especially those metastasized, are highly resistant to chemotherapeutic agents (Lowe and Lin, 2000; Johnstone et al., 2002). Apoptosis is regulated via different pathways (such as death-receptor and mitochondrial pathways) at different stages (such as the initiation, transduction, amplification, and execution stages), and mutations that disrupt each of these regulatory steps have been detected in tumor cells (Johnstone et al., 2002; Lowe and Lin, 2000). Different tumor types are known to have mutations that disable distinct steps in apoptosis, and different drugs can induce apoptosis via distinct pathways. Taken together, these facts suggest that disparate tumors may respond differentially to specific chemotherapeutic drugs and tumors that are resistant to certain drugs may otherwise be sensitive to other drugs. Therefore, combination chemotherapy that targets several apoptotic pathways simultaneously would overcome the problem of chemoresistance (Johnstone et al., 2002; Lowe and Lin, 2000). THL, which consists mainly of 14 Chinese herbal extracts, was shown here to induce apoptosis in a broad spectrum of human cancer cell lines. It is possible that THL contains various ingredients that induce apoptosis through a variety of independent pathways. This “chemotherapy cocktail” should be superior to conventional chemotherapeutic drugs because it can evade and

overcome the chemoresistance mechanisms that have befallen many conventional chemotherapeutic approaches. That THL can induce apoptosis via both death receptor- and mitochondrion-mediated pathways was demonstrated in Figures 3 and 4.

In summary, our present study demonstrates that THL can induce apoptosis in a variety of human cancer cell lines but not in normal human cells. THL treatment of H1299 cancer cells resulted in activation of caspase-8, -9 and -3, suggesting that THL may induce various apoptotic signals. These data together with our recent finding that THL has strong immunomodulatory effects (Sun et al., 2004) and other previous studies showing that the constituent herbs of THL have antioxidant, immunomodulatory, antimutagenic, cytotoxic and cytostatic effects strongly suggest that THL is a promising cancer chemotherapeutic or chemopreventive agent. This assertion was also supported by our animal experiments that showed that THL had antitumor activities in mice (data not shown).

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**FIG. 7.** THL could induce apoptosis of cancer cells efficiently in the presence of catalase. Bars indicate relative sub-G1 cell populations. In respective cancer-cell lines, the level of apoptosis (as indicated by sub-G1 cell population) induced by 1% (v/v) THL was set to 100%. The respective cancer-cell lines were preincubated with catalase (1000 units/mL; Sigma-Aldrich, Inc., St. Louis, MO) for 20 minutes before exposure to 1% (v/v) THL.

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