JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Mulberry Leaf Polyphenol Extract Induced Apoptosis Involving Regulation of Adenosine Monophosphate-Activated Protein Kinase/Fatty Acid Synthase in a p53-Negative Hepatocellular Carcinoma Cell

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ABSTRACT: The polyphenols in mulberry leaf possess the ability to inhibit cell proliferation, invasion, and metastasis of tumors. It was reported that the p53 status plays an important role in switching apoptosis and the cell cycle following adenosine monophosphate-activated protein kinase (AMPK) activation. In this study, we aimed to detect the effect of the mulberry leaf polyphenol extract (MLPE) on inducing cell death in p53-negative (Hep3B) and p53-positive (Hep3B with transfected p53) hepatocellular carcinoma cells and also to clarify the role of p53 in MLPE-treated cells. After treatment of the Hep3B cells with MLPE, apoptosis was induced via the AMPK/PI3K/Akt and Bcl-2 family pathways. Transient transfection of p53 into Hep3B cells led to switching autophagy instead of apoptosis by MLPE treatment. We demonstrated that acridine orange staining and protein expressions of LC-3 and beclin-1 were increased in p53-transfected cells. These results implied induction of apoptosis or autophagy in MLPE-treated hepatocellular carcinoma cells can be due to the p53 status. We also found MLPE can not only activate AMPK but also diminish fatty acid synthase, a molecular target for cancer inhibition. At present, our results indicate MLPE can play an active role in mediating the cell death of hepatocellular carcinoma cells and the p53 might play an important role in regulating the death mechanisms.

KEYWORDS: *mulberry leaf polyphenol extract, p53-negative Hep3B cell, apoptosis, AMP-activated protein kinase, fatty acid synthase*

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of liver cancer. There are many factors involved in hepatoma, such as aflatoxin B1, alcohol, hepatitis virus, and fat.[1−4] According to the possible molecular mechanisms, previous studies indicated that p53 mutation is an important factor involved in liver malignancy. P53 is a well-known tumor suppressor which can induce apoptosis via regulation of the Bcl-2 family proteins.[5−7] It was shown that the epoxide product metabolized from aflatoxin B1 binds to guanine and causes p53 tumor suppressor gene mutation and then falls from its tumor inhibition function.[2] The other study showed that hepatitis B virus (HBV) can cause DNA recombination and inactivate the p53 gene in liver cells.[4] Recently, growing evidence has shown p53 plays an important role in the liver tumor development.[8,9]

The adenosine monophosphate-activated protein kinase (AMPK) system is an energy sensor in eukaryotic cells to control the switching of ATP-consuming processes.[10,11] It is reported that the AMPK pathway is linked to tumor growth and proliferation via regulation of the mTOR pathway and also activated AMPK decreases the activity and/or expression of lipogenic enzymes, such as fatty acid synthase (FASN) in hepatoma.[12,13] FASN, a 250 kDa cytosolic multienzyme catalyzing eukaryotic de novo fatty acid biogenesis, localizes in cancer cell culture supernatants and in the blood of cancer patients and is known as a tumor promotion molecule in some studies.[14−17] High levels of extracellular FASN have recently been found in supernatants from hepatitis C virus-infected liver cells.[13] In 2009, Huang et al. demonstrated that the p53 status seems to play a decision switch between apoptosis and growth arrest following AMPK activation. (−)-Epigallocatechin-3-gallate blocked the progression of the cell cycle in p53-positive hepatoma cells but induced apoptosis in p53-negative hepatoma cells.[12] It still remains unclear whether some undefined pathways are controlled by p53 as AMPK activation.

Several studies indicated that edible phytochemicals have antioxidative, anti-inflammatory, and antitumor effects, contributing to induce cancer cells death via inducing apoptosis with low side effects for humans.[18−20] Mulberry leaf, the leaf of *Morus alba*, commonly used in the silkworm diet, has also been used in edible foods. It contains a lot of functional components, including flavonoid, which is known as a powerful polyphenol and antioxidant.[21] In previous reports, dietary mulberry leaf showed multiple functions, such as a wild spectrum of antimicrobial[22] and antioxidant and anti-inflammatory functions,[23] activity against atherosclerosis[24,25] and diabetes mellitus,[26,27] neuroprotective functions,[28] and DOPA oxidase inhibition and antityrosinase activity.[29,30] The polyphenols contained in mulberry leaf also showed the ability to inhibit cancer cell proliferation, invasion, and metastasis.[21]

Received: April 5, 2012
Revised: June 7, 2012
Accepted: June 7, 2012
Published: June 7, 2012

dx.doi.org/10.1021/jf302183x | *J. Agric. Food Chem.* 2012, 60, 6891−6898

In this study, we aimed to examine the cytotoxic effects induced by mulberry leaf polyphenol extract (MLPE) in cancer cells. Briefly, HCC cell lines, including p53-negative Hep3B cells, p53-transfected Hep3B cells, and p53-positive HepG2 cells, were treated with MLPE to detect its effects in inducing cell death. Furthermore, we examined the expression of AMPK and FASN and also clarified whether the p53 status plays an important role under these circumstances.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma Co. (St. Louis, MO) or Bio-Rad (Hercules, CA) unless otherwise noted. The Bcl-xL, Bcl-2, Bax, Bid, AMPK-α, and p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p-AMPK (Thr172), p-p53 (Ser15), and FASN antibodies were purchased from Cell Signaling Technology (Denver, MA). Anti-β-actin and HRP-conjugated secondary antibodies were obtained from Sigma.

Preparation of MLPE and Its Analysis by HPLC. The mulberry leaves were collected in Dadu Township, located in central Taiwan. The procedures of the extraction of mulberry leaves were described by Yang et al. in 2011.[31] After MLPE was extracted, the presence and proportion of the main constituents of MLPE were then analyzed by a Hitachi HPLC system (Hitachi, Danbury, CT). MLPEs were filtrated by a 0.22 μm filter before used in cell culture.

Cell Line Culture. Cell lines used in this research were purchased from the Bioresource Collection and Research Center (BCRC; Food Industry Research and Development Institute, Hsinchu, Taiwan) and American Type Culture Collection (ATCC; Rockville, CT). Human hepatocellular carcinoma Hep3B (BCRC 60434) and hepatoblastoma HepG2 (BCRC 60025) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL). The normal liver cell line, Chang liver (ATCC, CCL13), was maintained in basal medium Eagle (BME; GIBCO-BRL). The human gastric adenocarcinoma AGS cell line (BCRC 60102) and human colonic adenocarcinoma Ht29 cell line (ATCC HTB-38) were maintained in F-12 nutrient mixture medium (GIBCO-BRL). The human breast carcinoma cell line MCF-7 (BCRC 60436) was maintained in DMEM (GIBCO-BRL). All cell lines were cultured in medium supplemented with 10% fetal bovine serum (FBS), antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and 2 mM glutamine at 37 °C under 5% CO_2.

Cell Viability (MTT Assay). Cells were seeded on 24-well plates at a density of 7×10^4 cells/well and treated with the indicated concentrations of MLPE (dissolved in 50% ethanol) for 24 h. After incubation, the cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 5 mg/mL MTT solution was added to each well followed by incubation for 4 h at 37 °C. After washing with PBS, the purple-blue formazan was dissolved in 1 mL of 2-propanol, and the absorbance was determined at 563 nm. Cell viability was proportional to the amount of formazan.

Analysis of the Cell Cycle by Flow Cytometry. Flow cytometric analysis of Hep3B cells was performed using a FAScan for the indicated concentration of MLPE used to treat the culture. Thereafter, the cells were washed twice with Dulbecco's phosphate-buffered saline (PBS) solution. Then the cell suspension was centrifuged at 1500 rpm for 5 min at room temperature. Decanting of all the supernatants was followed by adding 1 mL of 70% methanol to the pellet. After incubation at −20 °C for at least 24 h, prior to the samples being analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ), 1 mL of cold propidium iodide (PI) stain solution (20 μg/mL PI, 20 μg/mL RNase A, and 0.1% Triton X-100) was added to the mixture followed by incubation for 15 min in darkness at room temperature. PI was excited at 488 nm, and the fluorescence signal was subjected to logarithmic amplification, with PI fluorescence (red) being detected above 600 nm. The cell cycle distribution is presented as the number of cells versus the amount of DNA as indicated by the intensity of fluorescence, and the extent of apoptosis was determined by counting the cells of DNA content below the sub-G1, G0/G1, S, and G2/M phases with CELLQuest version 3.3 software (Becton Dickinson). The percentage of hypodiploid cells (sub-G1 phase) over total cells was calculated and expressed as percent apoptosis.

Table 1. Composition and Characterization of Free Phenolic Compounds Contained in MLPE[a]

retention time (min)	assigned identity[b]	recovery (%)
14.41	PCA	3.52 ± 1.23
23.03	GC	1.21 ± 1.22
25.22	GCG	10.23 ± 2.05
26.18	Q3G	4.08 ± 0.66
27.23	CA	2.33 ± 0.67
32.50	R	5.69 ± 1.42
50.18	Q	11.73 ± 1.16
54.79	N	8.96 ± 1.64

[a]MLPE is extracted and then analyzed by HPLC chromatography. Recovery (%) means the percentage of polyphenols contained in MLPE after mulberry leaves were extracted by lyophilization and resuspended in solvents. The composition is compared with nine kinds of standard polyphenol HPLC peaks. [b]Key: PCA, protocatechuic acid; GC, gallocatechin; GCG, gallocatechin gallate; Q3G, quercetin 3-glucoside; CA, caffeic acid; R, rutin; Q,quercetin; N, naringenin.

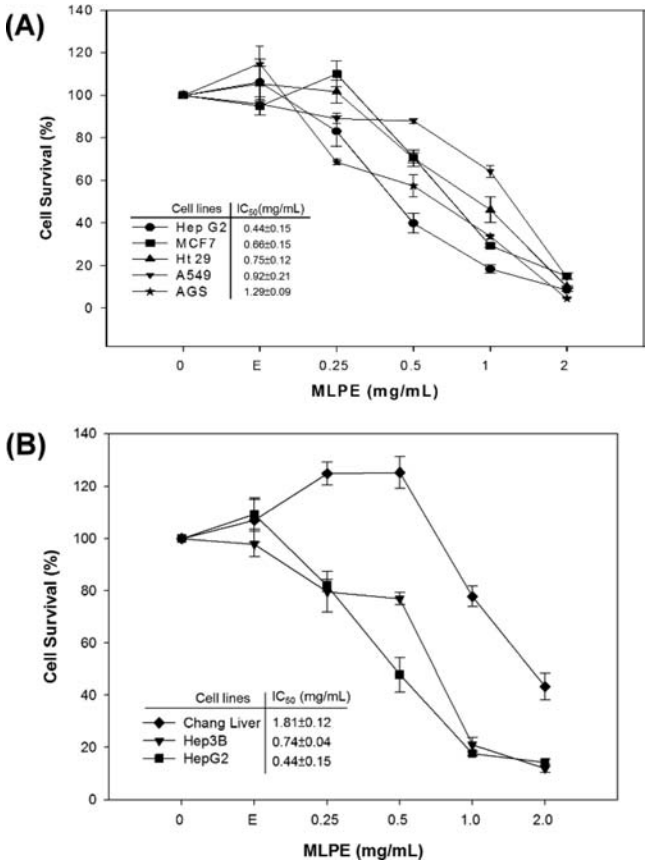

Figure 1. Effects of MLPE on the viability of (A) tumor cells and (B) hepatocellular carcinoma cells and normal liver cells (Chang liver). The data are expressed as a percentage of the control (dose 0) and presented as the mean ± SD from three independent experiments.

DAPI Stain. Changes in the cell morphological characteristics of apoptosis were examined by fluorescence microscopy of 4,6-diamidino-2-phenylindole (DAPI)-stained cells. The monolayer of cells was washed in PBS solution and fixed with 4% paraformaldehyde for 30 min at room temperature. The fixed cells were permeated with 0.2% Triton X-100 in PBS three times, incubated with 1 μg/mL DAPI for 30 min, and then washed with PBS three times. The apoptotic nuclei (intensely stained, fragmented nuclei and condensed chromatin) were examined on 400× magnification using a fluorescence microscope with a

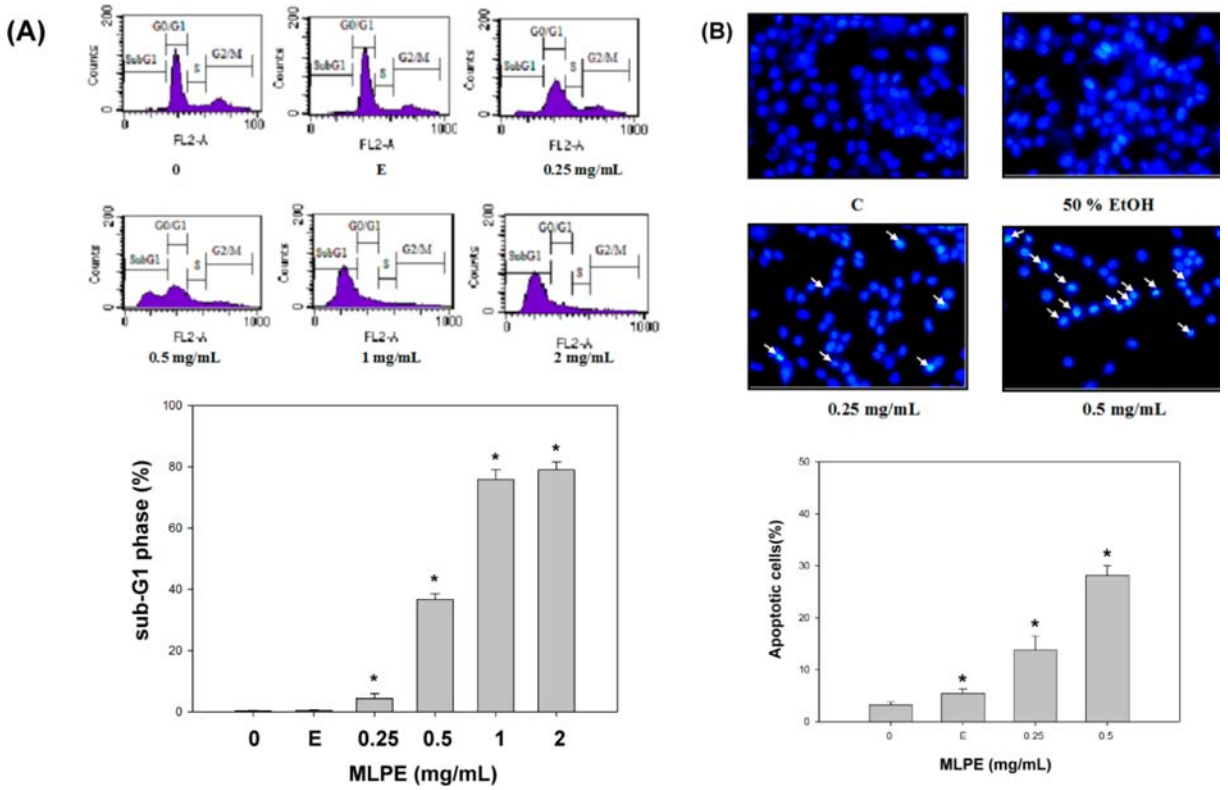

Figure 2. MLPE-induced apoptosis on Hep3B cells. (A) Flow cytometric analysis. Sub-G1 is defined as apoptotic cells. (B) DAPI staining. The arrow indicates apoptotic cells (magnification 400×). Apoptotic values were calculated as the percentage of apoptotic cells relative to the total number of cells in each random field (>100 cells) and are presented as the mean ± SD from three independent experiments. Results were statistically analyzed with Student's *t* test. An asterisk indicates $p < 0.05$ compared with the control. Group "E" means the ethanol control.

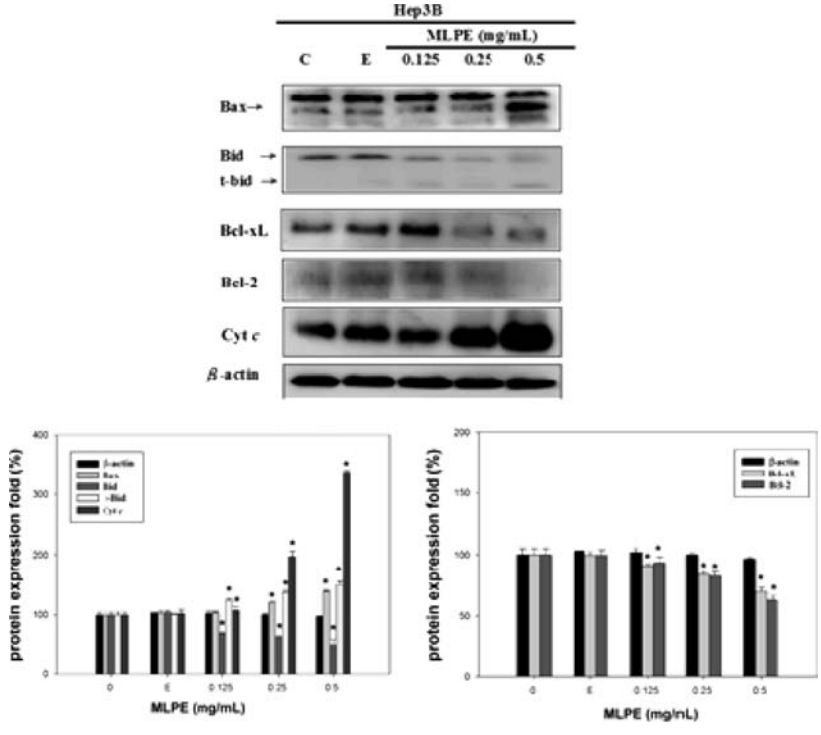

Figure 3. MLPE regulated the expression of the Bcl-2 family and cytochrome *c* (Cyt *c*) protein on Hep3B cells. Equal amounts of cellular lysates (50 μg) were analyzed by immunoblotting with anti-Bax, Bid, Bcl-xL, Bcl-2, Cyt *c*, and actin antibodies. Quantification data represent the average of three independent experiments as the mean ± SD, $n = 3$. An asterisk indicates $p < 0.05$ compared with the control.

340/380 nm excitation filter (Nikon DIAPHOT-300, Japan). The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted multiplied by 100. Three separate experiments were conducted, and at least 300 cells were counted for each experiment.

dx.doi.org/10.1021/jf302183x | J. Agric. Food Chem. 2012, 60, 6891−6898

Immunoblotting Assay. Total protein extracts were prepared in a lysis buffer (1% NP-40, 50 mM Tris base, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid, and 150 mM NaCl, pH 7.5) containing leupeptin (17 μg/mL) and sodium orthovanadate (10 μg/mL). The protein concentration was measured using a Bradford protein assay kit. Equal amounts of protein samples (50 μg) were subjected to SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk powder with 0.1% Tween-20 in Tris-buffered saline (TBS) and then incubated with the primary antibody at 4 °C overnight. Afterward, the membranes were washed three times with TBS Tween-20 (TBST) and incubated with the secondary antibody conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). The membranes were extensively washed with TBST, and the reactive signal was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, United Kingdom). β-Actin expression was used as the internal control. Band detection was thereafter revealed by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed to FUJFILM LAS-3000 (Tokyo, Japan). The protein quantity was determined by densitometry using FUJFILM-Multi Gauge V2.2 software.

Transient Transfection of p53. Liposome-mediated transfection was performed using Lipofectamine (Invitrogen, Carlsbad, CA) of Hep3B cells with expression vectors for constitutively active p53. Briefly, the Hep3B cells were plated onto six-well plates overnight, and transfections were carried out on the cells at 70–80% confluence of the cells the next day using lipofectamine reagent according to the manufacturer's instructions. Briefly, lipofectamine (10 μL) and DNA (8 μg) were diluted in 0.5 mL of DMEM without antibiotics and serum followed by equilibration at room temperature for 45 min after mixing. The lipofectamine–DNA complex was added to the Hep3B cells followed by incubation for 6 h. The cells were then washed with PBS and replenished with DMEM containing 10% serum. At 12 h after transfection, the cells were incubated with different concentrations of MLPE for 24 h and then expanded for further studies.

Statistical Analysis. Data were analyzed using an unpaired t test and are represented as means ± standard deviation (SD). Statistical differences were evaluated using the unpaired t test and considered significant at the $p < 0.05$ level. All data collected were analyzed using an unpaired t test after one-way analysis of variance (ANOVA) testing showed a significant difference among all the groups ($p < 0.001$).

RESULTS

Mulberry Extract Is Rich in Polyphenols. We checked the polyphenol components in mulberry leaf by HPLC to ensure the quality and quantity of polyphenols in mulberry leaf. MLPE has no obvious difference compared with that of Yang prepared in 2011.[31] Table 1 shows that protocatechuic acid (3.52%), gallocatechin (1.21%), gallocatechin gallate (10.23%), caffeic acid (2.33%), rutin (5.69%), quercetin (11.73%), and naringenin (8.96%) are contained in the composition of MLPE.

Cytotoxicity Induced by MLPE in Various Tumor Cell Lines and HCC Cell Lines. The cytotoxicity of MLPE to cells was measured by MTT assay. The various cells were cultured and treated with 0, 0.25, 0.5, 1.0, and 2.0 mg/mL MLPE for only 24 h. As shown in Figure 1A, various kinds of tumor cells decreased in survival rate after being treated with MLPE. The IC_{50} values of these cells were calculated as follows: HepG2, 0.44 mg/mL; MCF-7, 0.66 mg/mL; Ht29, 0.75 mg/mL, A549, 0.92 mg/mL; AGS, 1.29 mg/mL. The results indicate that polyphenols in mulberry leaf can induce cytotoxicity in cancer cells regardless of the mechanism. The ingredients existing in mulberry leaf might be used in antitumor therapy. To detect the cytotoxicity in normal liver cells and hepatocellular cells, Chang liver (normal liver cell line), Hep3B, and HepG2 were conducted to prove it. The results in Figure 1B indicate both of the liver cancer cells were sensitive to MLPE and decreased in survival rate, but the normal liver cells were not affected significantly. Interestingly, our results show that the normal liver cells treated with low doses

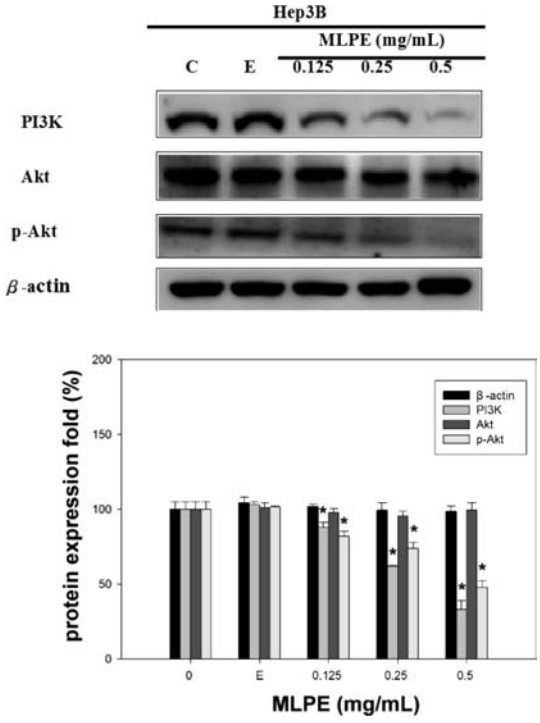

Figure 4. MLPE regulates the expression of PI3K/Akt protein on Hep3B cells. Equal amounts of cellular lysates (50 μg) were analyzed by immunoblotting with anti-PI3K, Akt, p-Akt, and actin antibodies. Quantification data represent the average of three independent experiments ± SD, $n = 3$. An asterisk indicates $p < 0.05$ compared with the control.

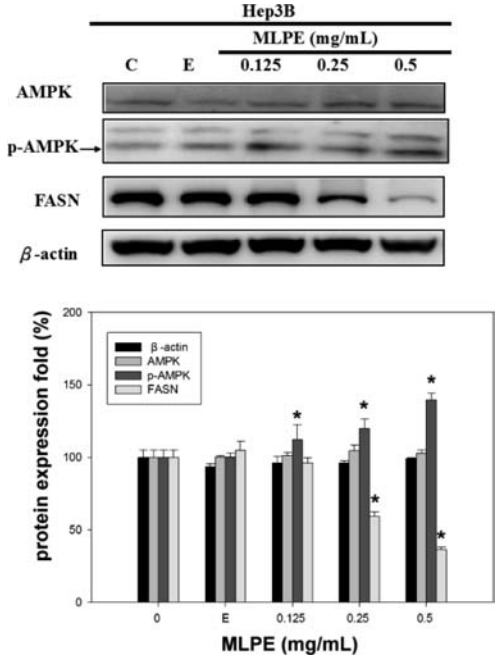

Figure 5. MLPE regulates the expression of AMPK/FASN protein on Hep3B cells. Equal amounts of cellular lysates (50 μg) were analyzed by immunoblotting with anti-AMPK, p-AMPK, FASN, and actin antibodies. Quantification data represent the average of three independent experiments ± SD, $n = 3$. An asterisk indicates $p < 0.05$ compared with the control.

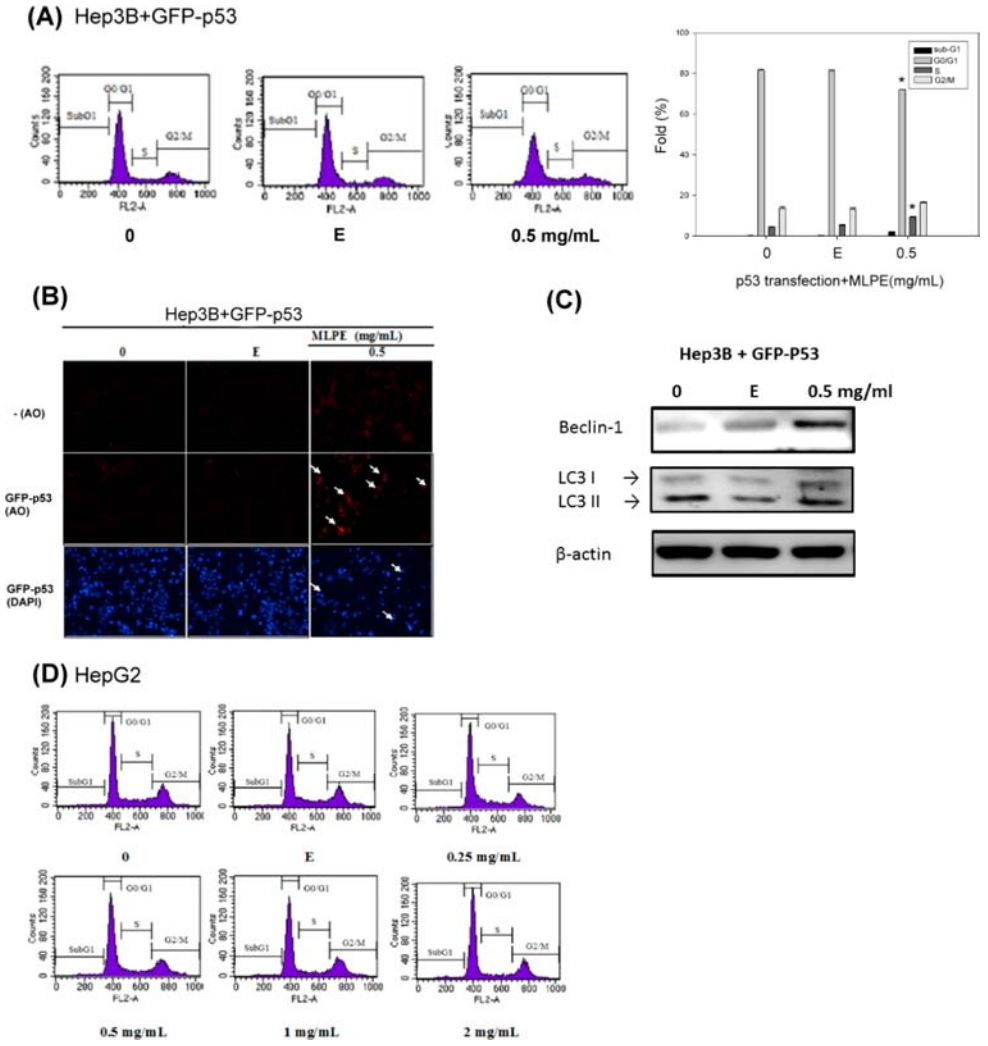

Figure 6. MLPE induces autophagy but not apoptosis in p53-positive HCC cells. (A) Flow cytometric analysis in p53-transfected Hep3B cells. Quantification data show the average of three independent experiments ± SD, $n = 3$. An asterisk indicates $p < 0.05$ compared with the control. (B) AO/DAPI stain in p53-transfected Hep3B cells shown at 100× magnification. (C) Immunoblotting analysis of p53-transfected Hep3B cells. Equal amounts of cellular lysates (50 μg) were analyzed by anti-Beclin-1, LC3, and actin antibodies. (D) Flow cytometric analysis in HepG2 cells.

of MLPE (0.25 and 0.5 mg/mL) have slightly increased proliferative properties, indicating the protective role of MLPE in normal liver cells. On the other hand, IC_{50} values for MLPE in nonmalignant Chang liver cells were more than 1.8 mg/mL (Figure 1B).

MLPE-Induced Apoptosis in Hep3B Cells. To test the possibility of apoptosis induced by MLPE, we observed the effects of MLPE in Hep3B cells by detecting the nuclear condensation and the sub-G1 ratio in the cell cycle. Hep3B cells were treated with 0, 0.25, 0.5, 1, and 2 mg/mL MLPE for 24 h and subjected to flow cytometric analysis (Figure 2A) and DAPI staining (Figure 2B). The results showed the sub-G1 fractions were increased after treatment with 0.25 mg/mL MLPE in Hep3B cells (Figure 2A). The quantification of sub-G1 was about 80% in 1 and 2 mg/mL MLPE treatment. To further verify whether the MLPE-mediated decrease in cell growth was due to induction of apoptosis, cells were stained with DAPI. Nuclear condensation was observed in a dose-dependent manner after MLPE treatment (Figure 2 B). However, unlike the Hep3B cells, the influence of MLPE on HepG2 cells was very low in apoptotic cells (data not shown).

The Bcl-2 Family and Mitochondrial Pathway Are Involved in MLPE-Treated Hep3B Cells. It is well established that Bcl-2 family proteins have major effects on the apoptosis signaling pathway. Expression of the Bcl-2 family on the mitochondrial membrane will regulate the release of cytochrome *c* from mitochondria and then activate the caspase family and lead to apoptosis.[32−34] Since the apoptotic pathway is the major type of MLPE-induced cell death on Hep3B tumor cells, we observed the expression of proapoptotic proteins Bax and Bid and antiapoptotic proteins Bcl-xL and Bcl-2.[35] In Figure 3, protein-induced apoptosis such as with Bax and Bid (t-Bid) increased after treatment with MLPE, but the expression of Bcl-xL and Bcl-2 decreased accompanied by cytochrome *c* being released. These findings indicate that the Bcl-2 family is involved in the MLPE-induced apoptosis, and it might be mediated via increasing expression of Fas and cleaved caspase-3, -8, and -9 proteins.

MLPE Activated AMPK and Inhibited FASN in Hep3B Cells. In addition to the Bcl-2 family, proteins involved in the PI3K/Akt pathway can also regulate apoptosis. Phosphorylation of AMPK will inhibit the activation of PI3K and then Akt.[36] Phosphorylation of Akt inhibited the expression of proapoptotic member Bad and diminished the apoptosis pathway in HCC cells.[37] Figures 4 and 5 show the expression of p-AMPK (Thr172)

dx.doi.org/10.1021/jf302183x | J. Agric. Food Chem. 2012, 60, 6891−6898

was increased after treatment with MLPE in Hep3B cells and those of PI3K and p-Akt (Ser473) were reduced. Conceivably, MLPE-induced apoptosis of Hep3B cells is highly relative to the PI3K/Akt and Bcl-2 pathways.

Previous studies indicated that FASN is a tumor promotion molecule.[14−17] Wu et al. also showed that AMPK regulated FASN in hepatocellular carcinoma cells.[38] Our data shown in Figure 5 indicated that the activation of p-AMPK (Thr172) was increased and the expression of FASN was decreased after treatment with MLPE.

p53 Tumor Suppressor Protein Involved in the MLPE-Induced Apoptosis Pathway. When we investigated the molecules involved in MLPE-induced apoptosis in Hep3B cells, it was also found, unlike Hep3B cells, the p53-positive HepG2 cells did not have the same apoptotic pattern after treatment with MLPE (Figure 6D). Due to the major difference of p53 existing in HCC cells,[39] we further investigated whether p53 affects the type of death pathway. Flow cytometry analysis showed that transfection of p53 decreased apoptosis by treatment with MLPE in Hep3B cells (Figure 6A). To further identify autophagy in these cells, the cells were stained with acridine orange (AO) and analyzed by fluorescence microscopy. The data indicated an increased dotlike pattern of AO staining in p53-transfected cells (Figure.6B). Western blot analysis showed the autophagy markers Beclin-1 and LC3 were increased in p53-transfected Hep3B cells by treatment with MLPE (Figure 6C). This result implied that p53 might play a key molecule in switching the type of cell death after treatment with MLPE. Figure 7 shows the phosphorylation of p53 (Ser15) was increased in p53-transfected Hep3B cells, indicating p53 was working in this condition.

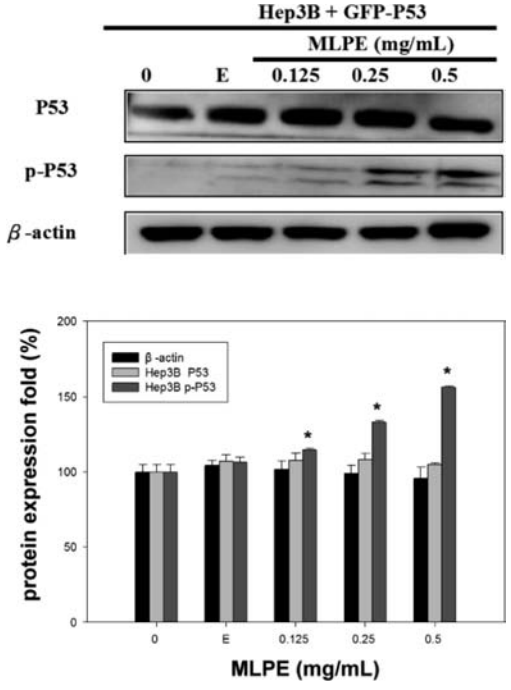

Figure 7. Immunoblot analysis of the p53 and p-p53 (Ser15) expression on Hep3B cells transiently transfected with GFP-P53. Equal amounts of cellular lysates (50 μg) were analyzed by immunoblotting with anti-p53, p-p53 (Ser15), and actin antibodies. Quantification data represent the average of three independent experiments ± SD, n = 3. An asterisk indicates $p < 0.05$ compared with the control.

DISCUSSION

Malignancy is one of the most difficult events to overcome currently. Many studies indicated that phytochemicals have antitumor effects. MLPEs containing polyphenols, such as quercetin and rutin, have been known to have antitumor ability. Previous studies showed both quercetin and rutin can inhibit proliferation of tumor cells and increase the survival rate in prostrate cancer cells and leukemia cells.[40,41] In this study, we found that MLPE contains quercetin (11.73%) and rutin (5.69%), which possess the ability to induce apoptosis in hepatocellular carcinoma cells. Our results showed MLPE could induce the deaths of many tumor cells, including breast carcinoma cells, lung cancer cells, gastric carcinoma cells, colonic adenocarcinoma cells, and hepatocellular carcinoma cells (Figure.2). This suggests that MLPE has broad effects on the deaths of different tumor cells, including HCC cells. Additionally, both Hep3B and HepG2 were sensitive to MLPE treatment and decreased in survival rate, and the normal liver cells have less cytotoxicity after MLPE treatment. This means the normal cells were affected less than tumor cells, and the cell death induced by MLPE is focused on tumor cells. This might be a valuable result for diminishing side effects of normal cells under tumor inhibition.

Apoptosis and autophagy are included in programmed cell death.[42,43]With regard to the antitumor drugs in reducing cancer cell viability, most researchers focused on the effect in apoptosis but less on that in autophagy. In this study, we found that MLPE induced cytotoxicity in both Hep3B (p53 null) and HepG2 (p53 wild type) cells (Figure 1B). Treatment of MLPE caused apoptosis in Hep3B cells (Figure 2A) and autophagy in HepG2 cells (data not shown). The different types of cell death are due to the p53 status in both Hep3B and HepG2 cells. The p53 tumor suppressor gene has been shown to induce apoptosis in response to many physiological stimuli.[44] However, a number of studies have shown that apoptosis can be induced in a p53-independent manner as well. Kang et al. reported that receptor for advanced glycation endproducts (RAGE) inhibits apoptosis by a p53-independent pathway during response to chemotherapeutic agents and sustains autophagy associated with increased Beclin 1−Vps34 interaction.[45] Some chemicals such as docosahexaenoic acid (DHA) have been reported to induce tumor cell death by apoptosis and to induce autophagy accompanied by p53 loss.[46] Our studies showed that MLPE possesses the ability to induce apoptosis in Hep3B cells in a p53-independent pathway. Transfection of p53 into Hep3B cells induced autophagy after treatment with MLPE, suggesting that apoptosis and autophagy are cross-regulated by the p53 status. Although the molecular effects of p53 in switching the death type are still unclear, we speculated p53 may have the key function in autophagy regulation in this system. For exploring the effective mechanism of cancer therapy, the role of p53 will be further elucidated in the future.

Our data showed the regulatory methods in antiapoptotic proteins (Bcl-2 and Bcl-xL) were down-regulated and in proapoptotic proteins (Bax and Bid) were up-regulated. The AMPK/PI3K/Akt pathway was also involved in the promotion of apoptosis. Recent studies have indicated that FASN not only regulated the metabolism of the fatty acid, but also was involved in the tumor cell progression. Inhibition of FASN will induce apoptosis, decrease tumor cell proliferation, suppress angiogenesis, and work against tumor metastasis.[14−17] Studies also indicated AMPK will inhibit the expression of FASN and then reduce the liver tumor cell growth.[38] Our data also indicated the

relationship between AMPK and FASN. We found that p-AMPK (Thr172) was increased in MLPE-treated-Hep3B cells accompanied by inhibition of FASN (Figure 5). Even in the Hep3B cells transiently transfected with p53, FASN was inhibited under the same conditions (data not shown). Since FASN is a main enzyme which participates in our HCC cell lines and can be regulated by phytochemicals, it may give a direction in hepatocellular carcinoma chemoprevention. Yang et al. also has shown that FASN is up-regulated in hepatitis C virus infection and part of the liver tumor or fatty liver induced by alcohol.[47] FASN may be one of the novel chemotherapeutic targets in alcohol or virus-related liver tumors regardless of whether p53 exists.

Taken together, our results showed that MLPE possesses the effect to induce apoptosis in p53-negative hepatocellular cells. Once p-AMPK is activated by MLPE, the expression of FASN is reduced and then Akt is decreased subsequently. This causes cytochrome *c* release from the mitochondrial membrane, and then the activated caspases induce the death of tumor cells. The mechanism indicated MLPE is a functional compound related to tumor chemoprevention. It is assumed Mulberry leaf possesses the ability to be used as a functional health food in cancer adjuvant therapy or chemotherapy.

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Funding

Funding for this research was provided by the National Science Council (NSC), Taiwan, under Grant NSC 99-2632-B-040-001-MY3.

Notes

The authors declare no competing financial interest.

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