Andrographolide induces autophagic cell death in human liver cancer cells through cyclophilin D-mediated mitochondrial permeability transition pore

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Introduction

Liver cancer is the third most leading cause of cancer death worldwide and about half of the patients with liver cancer require adjuvant therapy after surgical resection. Therefore, development of novel agents to eradicate cancer cells may constitute a viable approach to treat patients with liver cancer. Andrographolide, a diterpenoid lactone isolated from Andrographis paniculata, is known to possess potent antioxidant, anti-inflammatory, anitneoplastic and antiviral properties. In this study, we investigated the cytotoxic effect of andrographolide on human liver cancer cells and explored the cell death mechanism. Andrographolide induced a cell death distinct from apoptosis in multiple human liver cancer cells. The death was characterized by autophagy as evidenced by the accumulation of LC3 II and autophagosomes, and the formation of puncta GFP-LC3. This autophagy as well as cytotoxicity caused by andrographolide could be effectively prevented by 3-methyladenine (a chemical inhibitor of autophagy). Mechanistic study indicated that andrographolide induced autophagic cell death by disruption of mitochondrial membrane potential and elevation of reactive oxygen species, which were correlated with mitochondrial permeability transition pore (MPTP). These preclinical studies suggest that andrographolide might be a multitargeted inhibitor that performs its functions in a cell type-dependent manner.

Autophagy, a physiologic process that occurs in all eukaryotic cells, is characterized by the sequestration of bulk cytosolic components including unfolded proteins and membranous organelles, and delivery of the enclosed components to lysosomes for degradation (13). This serves as a temporary survival mechanism and favor tumor development (14). However, recent studies have shown that autophagy also has an active role in cell death (15,16). Autophagy or autopcell death, known as type II programmed cell death, is a response to various anticancer therapies in many kinds of cancer cells (17). Recently, several groups reported that some natural compounds could stimulate excess autophagy in cancer cells, which ultimately led to cell death (18–20). Therefore, natural compounds from medicinal herbs that trigger autopcell death may represent potent anticancer agents for the treatment of liver cancer.

In this study, we investigated the effects of andrographolide on human liver cancer cells and further examined the cell death mechanism. Our observations demonstrated that andrographolide-induced cytotoxicity was attributed to autophagy but not apoptosis in human liver cancer cells and that this autophagy-inducing activity was closely associated with the cyclophilin D-mediated mitochondrial permeability transition pore (MPTP). These preclinical studies suggest that andrographolide could be useful for the treatment of liver cancer.

Materials and methods

Reagents

Andrographolide (with >98% purity) was obtained from National Institutes for Food and Drug Control, China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamin123 (Rh123), cyclosporin A (CsA), bongkrekic acid (BA), 3-methyladenine (3-MA), dichlorodihydrofluorescein diacetate (DCFH-DA) and N-acetyl-L-cysteine (NAC) were purchased from Sigma. Pan-caspase inhibitor, z-VAD-fmk (z-VAD), was from R&D.

Cell lines and cell culture

The human liver cancer cell lines Huh-7, QGY-7703 and Bel-7402 were obtained from CBCTCCAS (the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China). The above cell lines were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Gibco), 100 units/ml penicillin, and 100 units/ml streptomycin in a humidified incubator with an atmosphere of 5% CO2 at 37°C.

Cell viability assay

Cell viability was measured by the MTT method as described previously (21). Briefly, cells were seeded into 96-well microtiter plates at a density of 5x10^3 cells/well. After 24 h of incubation, cells were treated with various concentrations of andrographolide. When incubated for the indicated times, cells were incubated with MTT (0.5mg/ml) for 4 h. The formazan precipitate was dissolved in 150 μl dimethyl sulfoxide, and the absorbance was detected at 490nm with a model E16800 profiector (Bio-Tek Instruments). Each test was performed in triplicate experiments.
siRNA duplex–lipofectamine was prepared by 6.0 μl of 10 μmol/l ATG5 siRNA, Cyclophilin D siRNA or control siRNA and 3.0 μl lipofectamine RNAiMAX (Invitrogen) in 500 μl siRNA Transfection Medium (Santa Cruz Biotechnology) according to the manufacturer’s instructions, and directly added into the media. Forty-eight hours after transfection, cells were collected. The knockdown of ATG5, or Cyclophilin D siRNA was measured by quantitative reverse transcription-PCR (qRT–PCR) and western blot.

**Statistical analysis**

Unless otherwise stated, data were expressed as mean ± SD. Student’s t-test was used to evaluate the statistical differences between the experimental values of two samples being compared. P < 0.05 was considered statistically significant.

**Results**

Andrographolide inhibited growth of Huh-7 cells independent of apoptosis

To identify the therapeutic potential of andrographolide, human liver cancer cell line Huh-7 was cultured with different concentrations of andrographolide for 24, 48 and 72 h, and then cell viability was determined by MTT assay. Andrographolide inhibited the growth of Huh-7 cells in a dose- and time-dependent manner (Figure 1A), with 50% inhibition (IC50) at 24, 48 and 72 h of 17.6, 11.3 and 8.1 μg/ml, respectively.

Because andrographolide has been reported to induce apoptosis in some cancer cells (23–25), we investigated whether andrographolide could induce apoptosis in Huh-7 cells by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. As shown in Figure 1B, there was no significant increase of apoptotic cells detected after 24 h andrographolide treatment. This finding was confirmed by caspase-3 activity analysis (Figure 1C). Similar results were also observed in other liver cancer QGY-7703 and Bel-7402 cells treated with andrographolide for 24 h (Figure 1D and 1E). Further studies revealed that there was no significant apoptosis observed after 48–72 h andrographolide treatment in Huh-7 (Supplementary Figure 1A and B, available at Carcinogenesis Online), QGY-7703 (Supplementary Figure 1C and D, available at Carcinogenesis Online) or Bel-7402 cells (Supplementary Figure 1C and D, available at Carcinogenesis Online).

Both TUNEL assay (Figure 1B and 1D; Supplementary Figure 1A and C, available at Carcinogenesis Online) and caspase-3 activity analysis (Figure 1C and 1E; Supplementary Figure 1B and D, available at Carcinogenesis Online) showed that doxorubicin did induce apoptosis in Huh-7, QGY-7703 and Bel-7402 cells, indicating that the pathway of apoptosis is intact in these cells. Taken together, our results indicated that andrographolide did not induce apoptosis in Huh-7, QGY-7703 or Bel-7402 cells.

Andrographolide induced autophagy in Huh-7 cells

Accumulating evidence indicates the importance of autophagy in tumor suppression (26,27). Autophagy or autophagic cell death, as a response to various anticancer therapies in many kinds of cancer cells (28,29). To determine whether autophagy is involved in andrographolide-induced growth inhibition of Huh-7 cells, western blotting was first performed to detect the conversion of cytosolic LC3-I to lipidated, autophagosome-membrane-bound LC3II, which is a specific marker for autophagosome formation (30). As shown in Figure 2A, compared with untreated group, andrographolide induced significantly higher amount of LC3II expression in Huh-7 cells after 24 h of treatment. Similar phenomena were also observed in QGY-7703 and Bel-7402 cells (Figure 2A). To confirm the characteristics of autophagy elicited by andrographolide, pucta GFP-LC3 and accumulation of autophagosomes in Huh-7 cells were investigated by fluorescence microscopy and transmission electron microscopy. Fluorescence microscopy analysis showed that a punctate pattern of LC3 was observed in andrographolide-treated Huh-7 cells, whereas a diffused distribution of LC3 was found in control cells (Figure 2B).

Moreover, a significant increase in the percentage of autophagic cells (cells with GFP-LC3 dots) was found in 8 and 16 μg/ml andrographolide-treated Huh-7 cells (Figure 2C).

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also found in QGY-7703 and Bel-7402 cells (Figure 2C). In addition, Huh-7 cells showed cytoplasmic accumulation of autophagosomes, a morphological correlate of autophagy, as determined by transmission electron microscopy, after andrographolide treatment (Figure 2D). Taken together, these results demonstrated that andrographolide induced autophagy in Huh-7 cells.

Autophagy inhibition attenuated andrographolide-induced cytotoxicity

To determine whether the cytotoxicity of andrographolide toward Huh-7 cells was caused by autophagy, we further used 3-methyladenine (3-MA, a chemical inhibitor of autophagy) to examine the effect of andrographolide on autophagy and cell viability in Huh-7 cells. As illustrated in Figure 3A, andrographolide-induced accumulation of GFP-LC3 vacuoles (cells with GFP-LC3 dots) was markedly reduced by pretreatment with 2 mM 3-MA for 2 h, whereas the caspase inhibitor z-VAD-fmk (z-VAD) failed to change GFP-LC3 vacuoles formation in Huh-7 cells (Figure 3A). To investigate whether inhibition of autophagy affected the cytotoxicity of andrographolide, Huh-7 cells were treated with 16 μg/ml andrographolide for 24 h in the presence of 2 mM 3-MA. As shown in Figure 3B, 3-MA significantly attenuated andrographolide-induced cytotoxicity in Huh-7 cells. In contrast, the caspase inhibitor z-VAD failed to reverse andrographolide-induced cytotoxicity. ATG5 has been characterized as a ubiquitin-like protein involved in autophagosome formation (31). We hypothesized that the suppression of ATG5 expression will decrease andrographolide-induced autophagy and cytotoxicity. In Huh-7 cells, the expression of ATG5 was reduced following transfection of siRNA against ATG5 (Figure 3C). The level of andrographolide-induced cell death was also attenuated by ATG5 siRNA (Figure 3E). Altogether, these data indicated that andrographolide induced cytotoxicity of Huh-7 cells through an autophagic mechanism.
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Fig. 2. Andrographolide-induced autophagy. (A) Western blotting was performed to determine LC3II accumulation in Huh-7, QGY-7703 and Bel-7402 cells after treatment with 0–16 μg/ml andrographolide for 24 h. Actin (β-actin) was used as internal control to ensure that equal amounts of proteins were loaded in each lane. (B) GFP-LC3 puncta was detected in Huh-7 cells after treatment with 0–16 μg/ml andrographolide for 24 h as described in Materials and methods. (C) The percentage of cells with GFP-LC3 dots (punctate GFP-LC3) was determined in Huh-7, QGY-7703 and Bel-7402 cells after treatment with 0–16 μg/ml andrographolide for 24 h as described in Materials and methods. Cells were visualized by fluorescence microscopy, and the GFP-expressing cells were counted as punctate or diffuse. The results are means ± SD of three independent experiments (*P < 0.05 versus control). (D) Autophagic vesicles or autophagosomes in control or 16 μg/ml andrographolide-treated Huh-7 cells were observed by electron microscopy after 24 h. The bottom picture enlargement showing clearer autophagic vesicles. A0, andrographolide 0 μg/ml; A4, andrographolide 4 μg/ml; A8, andrographolide 8 μg/ml; A16, andrographolide 16 μg/ml. Data of column represent means ± SD of three independent experiments (*P < 0.05 versus control).
Andrographolide-induced autophagy is associated with a loss of MMP and an increased ROS production

Recent studies reported that mitochondrial events were involved in the autophagy process (32,33). Therefore, we next investigated the effect of andrographolide on the integrity of mitochondria. As shown in Figure 4A, incubation of Huh-7 cells with andrographolide caused a substantial decrease of MMP in a time-dependent manner, as revealed by staining of the cells with a transmembrane potential-sensitive dye rhodamine-123 (Rh 123) and analyzed with flow cytometry analysis. To further determine the relationship between autophagy and disruption of MMP, we used an autophagy inhibitor 3-MA to examine the effect of andrographolide on MMP in Huh-7 cells. As illustrated in Figure 4A, the autophagy inhibitor 3-MA failed to reverse andrographolide-induced MMP decrease in Huh-7 cells. Moreover, ATG5 siRNA also failed to attenuate andrographolide-induced MMP loss (Figure 4A). Opening of the MPTP may result in dissipation of MMP (34), therefore we next used CsA (a MPTP inhibitor) to determine the effect of andrographolide on MMP in Huh-7 cells. Interestingly, andrographolide-induced collapse of MMP was largely prevented by a 2h pretreatment with 5 μM CsA (Figure 4A). To elucidate if this phenomenon was due to a direct effect on mitochondria, we isolated mitochondria from Huh-7 cells and measured changes in absorbance at 540nm, which is an indicator of mitochondrial swelling as a result of MPTP opening (22). Andrographolide induced a significant decrease in the absorbance at 540nm (Figure 4B), which can be largely blocked by CsA, indicating that andrographolide induced CsA-sensitive mitochondrial permeability transition. Since CsA can specifically interact with cyclophilin D (the MPTP component) and affects the pore permeability (35,36), these data suggested a possibility that cyclophilin D might be a target of andrographolide.

Recent investigations have reported that generation of ROS is associated with disruption of MMP (37). Therefore, we analyzed the production of intracellular ROS in andrographolide-treated Huh-7 cells by flow cytometry using DCFH-DA fluorescence dye. As shown in Figure 4C, compared with untreated cells (the control group), andrographolide caused a dramatic increase of ROS generation in Huh-7 cells in a time-dependent manner. This ROS increase could be largely suppressed by 2h pretreatment with 5 μM CsA, whereas the autophagy inhibitor 3-MA (Figure 4C) or ATG5 siRNA (Figure 4D) failed to suppress andrographolide-induced ROS increase, suggesting that the increase of ROS generation might be a consequence of andrographolide-induced disruption of MMP.
leading to respiratory dysfunction and electron leakage from the respiratory chain.

Cyclophilin D played an important role in andrographolide-induced autophagic cell death. Because andrographolide caused a significant increase of ROS, which can be suppressed by CsA (the cyclophilin D targeted compound), we further determined the role of ROS generation and the significance of cyclophilin D in mediating andrographolide-induced autophagy and cytotoxicity. As illustrated in Figure 5A, treatment with 16 μg/ml andrographolide resulted in a significant increase of autophagy in Huh-7, QGY-7703 and Bel-7402 cells (Figure 5A). Accordingly, a dramatic cell death was also observed in these andrographolide-treated cells (Figure 5B). This autophagy increase and massive cell death could be largely prevented by 2h pretreatment with 5 μM CsA (Figure 5A and 5B). Interestingly, preincubation of cells with antioxidant NAC (1 mM) failed to suppress andrographolide-induced autophagy (Figure 5C; Supplementary Figure 2A, available at Carcinogenesis Online) or cell death (Figure 5D; Supplementary Figure 2B, available at Carcinogenesis Online), although ROS generation caused by andrographolide was reduced by the antioxidant NAC (Figure 4C; Supplementary Figure 2C, available at Carcinogenesis Online). These results suggest that the increase of cellular ROS was not a critical event for andrographolide-induced autophagic cell death and might be merely an indication of mitochondrial dysfunction caused by andrographolide. Moreover, pretreatment of cells with 5 μM BA, an inhibitor of adenine nucleotide translocase (ANT, also a component of MPTP) (38), did not suppress andrographolide-induced autophagy (Figure 5E) or cell death (Figure 5F). Taken together, these data suggest that cyclophilin D might play a key role in mediating andrographolide-induced autophagic cell death.
To further determine the critical role of cyclophilin D, we used siRNA to knock down the endogenous cyclophilin D and then evaluated its effect on andrographolide-induced autophagic cell death. As shown in Figure 6A, transfection of cells with Cyclophilin D siRNA led to a dramatic reduction of cyclophilin D protein levels in Huh-7, QGY-7703 and Bel-7402 cells, whereas the scrambled RNA did not alter cyclophilin D expression. Importantly, andrographolide-induced autophagy (Figure 6B) and cell death (Figure 6C) were largely suppressed by knockdown of Cyclophilin D, further confirming the key role of cyclophilin D in mediating andrographolide-induced autophagic cell death.

Discussion

This study showed that treatment with andrographolide-induced autophagy but not apoptosis in human liver cancer Huh-7, Bel-7402 and QGY-7703 cells. Moreover, andrographolide-induced autophagy as well as cytotoxicity was largely attenuated by autophagy inhibitor 3-MA or ATG5 siRNA. We further demonstrated that MPTP played a critical role in andrographolide-induced autophagy. More importantly, cyclophilin D probably mediated the cytotoxic action of andrographolide by triggering the opening of MPTP.
Andrographolide has been shown to induce apoptosis in various types of cancer cells (24, 25, 39, 40). However, we did not detect any increase of apoptosis signal (TUNEL or caspase-3 assay) in andrographolide-treated human liver cancer Huh-7, QGY-7703 or Bel-7402 cells. The pan-caspase inhibitor z-VAD did not attenuate andrographolide-caused cell death. Taken together, these results indicated that andrographolide-induced cell death is not via caspase-dependent apoptosis in these cells. Rather, andrographolide was found to trigger autophagy instead of apoptosis in Huh-7, QGY-7703 and Bel-7402 cells. Autophagy is a process by which subcellular constituents are degraded in autophagosomes/autolysosome in response to stress (41). The significance of autophagy in antitumor therapeutics has not been clearly elucidated. To adapt adverse conditions induced by stress from anticancer therapies, cancer cells may trigger an autphagic response that promotes a portion of the cytoplasm and organelles into autophagic vesicles as part of the survival response to stress (14, 42).

In this case, autophagy may be a survival mechanism of cancer cells. On the other hand, some anticancer agents, including arsenic trioxide and rapamycin, have been reported to stimulate excess autophagy, which ultimately led to a caspase-independent cell death (17). In this regard, autophagy might be a crucial mechanism of cancer cell death by these agents. Whether autophagy triggered by andrographolide in Huh-7 cells is a survival mechanism or a cell death mechanism was elucidated in this study by applying 3-MA, a chemical inhibitor of autophagy. Our results indicated that 3-MA could reverse the cytotoxic effect of andrographolide, suggesting that andrographolide induces an autophagic cell death. These findings further validated by silencing of CypD, a key molecule involved in autophagosome formation. The ability of andrographolide to trigger multiple death pathways suggests its effectiveness and versatility in killing cancer cells.

Recently, caspase-independent autophagic cell death has been reported to associate with alteration of ROS (43). Consistent with these findings, intracellular ROS was elevated after being exposed to andrographolide. Furthermore, this study suggests that the mechanism of andrographolide-induced cytotoxicity appeared not directly through ROS caused oxidative damage, but probably through activating MPTP, leading to mitochondrial dysfunction and autophagic cell death. Several evidence could support this conclusion. First, antioxidant NAC effectively blocked cellular ROS production, but did not prevent andrographolide-triggered autophagy or cell death, suggesting ROS may not play a critical role in andrographolide-induced cytotoxicity. Second, andrographolide caused a dramatic decrease of MMP, which could not be blocked by 3-MA or ATG5 siRNA, whereas CsA, an agent that prevents the opening of MPTP by binding to cyclophilin D (36), could effectively block the collapse of MMP, ROS elevation, autophagy and cell death. Third, knockdown of Cyclophilin D by siRNA abrogated andrographolide-induced autophagy and cell death. Together, these findings indicated that the cytotoxicity of andrographolide was closely associated with cyclophilin D, which seemed to play an important role in andrographolide-induced autophagic cell death. Moreover, the observation that BA (a chemical inhibitor of ANT, another MPTP component) (38) failed to attenuate andrographolide-induced autophagy or cell death further suggests that cyclophilin D might be specific for andrographolide-induced cytotoxicity.

In summary, our study revealed that andrographolide induced a death pathway characterized by autophagy, in which cyclophilin D mediated the cytotoxic action by triggering the opening of the mitochondrial permeability transition pore. Thus, andrographolide may represent a promising novel targeted agent in the prevention and treatment of liver cancer. Further investigation of andrographolide in mouse models will contribute to the additional understanding of its in vivo activity toward malignant cells.
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Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

Funding

National Natural Science Foundation of China (31000775); National Key Technology R&D Program of China during the 12th Five-Year Plan Period (No. 2012BAD33B08), Research Fund for the Doctoral Program of Higher Education of China (No. 2010326120006) and Key Project of Chinese Ministry of Education (No. 210086).

Conflict of Interest Statement: None declared.

References


Received April 19, 2012; revised June 23, 2012; accepted July 29, 2012