Punicalagin induces apoptotic and autophagic cell death in human U87MG glioma cells

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**Aim:** To investigate the effects of punicalagin, a polyphenol isolated from *Punica granatum*, on human U87MG glioma cells in vitro.

**Methods:** The viability of human U87MG glioma cells was evaluated using MTT assay. Cell cycle was detected with flow cytometry analysis. The levels of Bcl-2, cleaved caspase-9, cleaved poly(ADP-ribose) polymerase (PARP), phosphor-AMPK and phosphor-p27 at Thr198 were measured using immunoblot analyses. Caspase-3 activity was determined with spectrophotometer. To determine autophagy, LC3 cleavage and punctate patterns were examined.

**Results:** Punicalagin (1–30 µg/mL) dose-dependently inhibited the cell viability in association with increased cyclin E level and decreased cyclin B and cyclin A levels. The treatment also induced apoptosis as shown by the cleavage of PARP, activation of caspase-9, and increase of caspase-3 activity in the cells. However, pretreatment of the cells with the pan-caspase inhibitor z-DEVD-fmk (50 µmol/L) did not completely prevent the cell death. On the other hand, punicalagin treatment increased LC3-II cleavage and caused GFP-LC3-II-stained punctate pattern in the cells. Suppressing autophagy of cells with chloroquine (1–10 µmol/L) dose-dependently alleviated the cell death caused by punicalagin. Punicalagin (1–30 µg/mL) also increased the levels phosphor-AMPK and phosphor-p27 at Thr198 in the cells, which were correlated with the induction of autophagic cell death.

**Conclusion:** Punicalagin induces human U87MG glioma cell death through both apoptotic and autophagic pathways.

**Keywords:** punicalagin; human glioma; autophagy; apoptosis; microtubule-associated protein light chain 3; AMPK; p27; z-DEVD-fmk; chloroquine
to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is further recruited to the membranes of the autophagosomes. The amount of LC3-II present correlates with the number of autophagosomes\textsuperscript{[10]}. Atg7 is the homologue of an ubiquitin activating enzyme, and the other Atg proteins function during vesicle formation. Autophagy can be regulated by many pathways\textsuperscript{[11,12]}. The classical pathway acts through the class III phosphatidylinositol 3-kinase (PI3K-III), which modulates autophagy via the mammalian target of rapamycin (mTOR)\textsuperscript{[13,14]}. Inhibition of the mTOR-dependent signaling pathway by rapamycin or by the energy sensing AMP-activated protein kinase (AMPK) causes autophagy in both yeast and mammalian cells\textsuperscript{[13,14]}. Active AMPK has been shown to phosphorylate the cyclin-dependent kinase inhibitor p27 (Kip1) at Thr 198, leading to an increase in p27 stability. Therefore, disrupted nutrient and energy metabolism is linked to cell-cycle progression through the AMPK/p27 signaling pathway\textsuperscript{[15]}. We demonstrated that punicalagin induced apoptosis through the activation of the caspase-9/caspase-3 cascade and through the cleavage of poly(ADP-ribose) polymerase (PARP) in U87MG cells. We also showed that punicalagin increased the formation of autophagosomes and the accumulation of LC3-II. Because punicalagin increases the phosphorylation of AMPK and p27\textsuperscript{[16,17]}, it is possible that punicalagin induces autophagic cell death through the AMPK/p27 signaling pathway. The results of the present study indicate that punicalagin-induced cell death is mediated through both the apoptotic and autophagic pathways.

Materials and methods

Materials

Punicalagin was kindly provided by Dr Ta-chen LIN at the Central Taiwan University of Science and Technology in Taiwan. Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), glutaamine, gentamycin, penicillin, and streptomycin were purchased from Life Technologies (Gaithersburg, USA). Benzylxoycyanovinyl-Asp-Glu-Val-Ala-Asp (OMe) fluoromethyl ketone (z-DEVD-fmk) was obtained from Sigma-Aldrich Chemical Co (St Louis, USA). Antibodies specific to cyclins A, B, and E, Cdk2, p21\textsuperscript{[18]}, p27\textsuperscript{[19]}, PARP, Bcl-2, caspase-9, phosphorylated AMPK, total AMPK, LC3, and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, Santa Cruz, USA). Horseradish peroxidase-conjugated anti-rabbit IgG antibodies were purchased from Bio-Rad (Hercules, USA). Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim (Mannheim, Germany). The human U87MG cell line (ATCC Ng HTB-14) was purchased from the Institute of Food Sciences (Hsin-Chu, Taiwan, China).

Culture of human glioma cells and preparation of cell lysates

U87MG cells were cultured in DMEM supplemented with 13.1 mmol/L NaHCO\textsubscript{3}, 13 mmol/L glucose, 2 mmol/L glutamine, 10% heat-inactivated FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were attached to Petri dishes after 24 h incubation and were maintained in a humidified incubator with 5% CO\textsubscript{2} at 37°C. After reaching confluence, the cells were treated with various concentrations of punicalagin and incubated in a humidified incubator at 37°C for the indicated time intervals. At the end of the incubation, the cell lysates were lysed in lysis buffer containing 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EGTA, 1 mmol/L MgCl\textsubscript{2}, 1 mmol/L sodium orthovanadate, 1 mmol/L DTT, 0.1% mercaptoethanol, 0.5% Triton X-100, and the protease inhibitor cocktail, with final concentrations of 0.2 mmol/L PMSF, 0.1% aprotinin, and 50 μg/mL leupeptin. The lysates were stored at -70°C for further measurements.

Polyacrylamide gel electrophoresis, immunoblotting, and caspase-3 assay

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were electrotransferred onto a polyvinylidenefluoride (PVDF) membrane. After transfer, the PVDF membrane was washed once with phosphate-buffered saline (PBS) and twice with PBS plus 0.1% Tween 20 (PBST). The PVDF membrane was then blocked with blocking solution containing 5% nonfat dry milk in PBST for 1 h at room temperature. The PVDF membrane was then probed with primary antibodies in the blocking buffer, washed again in PBST, and subsequently incubated with peroxidase-conjugated anti-mouse IgG antibodies for 1 h. The results were visualized using a chemiluminescence kit (Amersham, USA). To determine the caspase-3 activity of the cells, 60 μL cell lysate (~5×10\textsuperscript{6} cells) was diluted with an equal volume of reaction buffer (40 mmol/L HEPES, pH 7.5, 20% glycerol, 4 mmol/L DTT, and 400 µmol/L Ac-DEVD-pNA substrate; Calbiochem, USA). The mixture was incubated at 37°C for 3 h, and the caspase activity was determined by measuring the optical density at 405 nm in a spectrophotometer.

MTT assay and determination of the autophagic LC3 punctate pattern

Cell viability, an indication of cytotoxicity, was evaluated using an MTT assay. Gliona cells grown on 150 mm plates were washed twice with PBS and resuspended in DMEM. The suspended cells were plated on 24-well plates at a density of 2×10\textsuperscript{5} cells/well and were treated with the indicated reagent(s) for 24 h. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to the medium at a final concentration of 1 mg/mL and incubated at 37°C for 4 h. Dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals, and the absorbency of each well was measured at 570 nm. To determine the punctate pattern of microtubule-associated protein light chain 3 (LC3), the cells were transfected with GFP-LC3 or EGF construct using the Lipopectum reagent (Invitrogen, USA), followed by treatment with 10 μg/mL punicalagin or the same volume of DMSO for 24 h. The cells were fixed with 4% paraformaldehyde, and fluorescence images were taken.
PI staining and flow cytometry

Cells were treated with different concentrations of punicalagin for 24 h, fixed with alcohol, and stained with 50 µg/mL propidium iodide (PI) to determine the DNA content. Apoptosis was detected using flow cytometry analysis.

Statistical analysis

The data are expressed as the mean±SEM of triplicate experiments. Comparisons between groups were made using Student’s t-test, and a P value <0.05 was considered statistically significant.

Results

Punicalagin inhibits growth of U87MG glioma cells

To determine whether punicalagin exerts anti-proliferative effects, U87MG cells were incubated with different concentrations of punicalagin, and the viable cell number was determined using an MTT assay. Treatment of cells with punicalagin resulted in a decrease in the number of viable cells after 24 or 48 h (Figure 1A, P<0.05). Because cell-cycle progression is regulated by the activity of cyclin/cyclin-dependent kinase (Cdk) complexes, uncontrolled expression of cyclins and/or Cdk inhibitors may lead to either tumorigenesis or cell-cycle arrest. We next examined whether punicalagin affected cyclin/cdk expression. Treatment of cells with punicalagin down-regulated the expression of cyclins A and B, but up-regulated cyclin E expression in a dose-dependent manner (Figure 1B). Punicalagin 10 µg/mL also induced p27\(^{kip}\), but not p21\(^{cip}\), expression in U87MG cells (Figure 1B). Altered expression of the cyclins and Cdk inhibitors were associated with cell-cycle arrest and an accumulation of sub-G\(_1\) cells as determined by flow cytometry (Figure 1C).

Punicalagin causes apoptotic cell death in U87MG cells

Apoptosis, the process of programmed cell death, is morphologically characterized by membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation, as well as changes in mitochondrial and caspase activities. To investigate whether punicalagin induced apoptosis in U87MG cells, apoptotic features, such as the expression of Bcl-2, the cleavage of caspase-9 and poly(ADP-ribose) polymerase (PARP), and the activation of caspase-3, were examined. When cells were treated with different concentrations of punicalagin (1, 3, 10, or 30 µg/mL), the abundance of Bcl-2 protein decreased, and the cleaved forms of caspase-9 and PARP increased (Figure 2A–2C); the cleavage of caspase-9 was also determined to occur in a time-dependent manner (Figure 2D). The progression of apoptosis was also reflected by an increase in caspase-3 activity (Figure 2E, P<0.05), as determined by incubation with the Ac-DEVD-pNA substrate.

Punicalagin induces autophagy in U87MG cells

Autophagy, also known as type II cell death, is a process used to maintain homeostasis between synthesis and degradation and involves the degradation of intracellular long-lived proteins or damaged organelles via the lysosomal machinery. To further investigate whether punicalagin alternatively induced cell death through a non-apoptotic pathway, U87MG cells were pretreated with the pan-caspase inhibitor z-DEVD-fmk prior to incubation with punicalagin. As shown in Figure 3A, punicalagin-induced cell death was not completely inhibited by pretreatment with z-DEVD-fmk, indicating that the cell death response was not fully derived from the activation of the caspases. To determine the possibility of punicalagin-induced autophagy, cells were treated with different concentrations of punicalagin, and the accumulation of microtubule-associated protein light chain 3 II (LC3-II, with a molecular weight of 16 kDa) was analyzed by immunoblot analysis (Figure 3B). When treated with 30 µg/mL punicalagin, the transient accumulation of the short half-lived LC3-I, converted from LC3-I (18 kDa), was observed in association with a decrease in the amount of LC3-I protein. On the other hand, U87MG cells expressing green fluorescence-LC3 fusion proteins (GFP-LC3) were used to obtain independent evidence that supports the conclusion that punicalagin triggered autophagy. As shown in Figure 3C, the punctate fluorescence pattern of GFP-LC3 observed in the punicalagin-treated cells is presumably due to the incorporation of the GFP-LC3 into autophagosomes; this pattern was not observed in the control cells, indicating the progression of autophagy.

Chloroquine protects U87MG cells from punicalagin-induced autophagic cell death

Chloroquine, a lysosomotropic agent, has been shown to increase the intra-lysosomal pH and destroy the lysosomal enzymes, which inhibits the fusion of the autophagosome with the lysosome, thereby suppressing autophagy\(^{[16, 17]}\). Chloroquine treatment exhibited a mild cytotoxic effect in U87MG cells (Figure 4A, P<0.05). To examine whether the inhibition of autophagy by chloroquine protects the cells from punicalagin-induced autophagic cell death, U87MG cells were pretreated with different concentrations of chloroquine for 30 min prior to incubation with 10 µg/mL punicalagin for 48 h. The results showed that chloroquine prevented the punicalagin-induced cell death (Figure 4B, P<0.05) and caused a more significant increase in the accumulation of LC3-II (Figure 4C).

Punicalagin increases AMPK and p27\(^{TIP28}\) phosphorylation

AMP-activated protein kinase (AMPK) has emerged as a critical regulator of autophagy, possibly through the regulation of the mTOR-dependent signaling pathway\(^{[18, 19]}\). Compound C, an inhibitor of AMPK, or a dominant-negative form of AMPK, have been reported to largely inhibit autophagy in mammalian hepatocyte, HT29, and HeLa cells\(^{[20]}\). We therefore investigated whether punicalagin-induced autophagy was associated with increased AMPK activity in U87MG cells. Activation of AMPK, as noted by increased phosphorylation, was observed when cells were treated with different concentrations of punicalagin for 15 min; this result was observed in a dose-dependent manner (Figure 5A). Furthermore, increased phosphorylation of p27 at threonine 198 has been implicated as an autophagic signal\(^{[19]}\). Our results showed that punicalagin...
elicited a dose-dependent increase in p27 phosphorylation at Thr 198 (Figure 5B), supporting the hypothesis that the induction of autophagy by punicalagin is through the LKB1-AMPK-p27 signaling pathway.

Discussion

Punicalagin is a polyphenol found in many fruits, vegetables, and derived products such as wine and tea. The antiproliferative and pro-apoptotic effects of pomegranates have been attributed to the presence of polyphenols in the fruit’s extract. The anticancer effects of punicalagin have been shown in many cancer cell lines. Our results are in line with many previous reports that have shown the association of the punicalagin’s antiproliferative effects with the up-regulation of cyclin E and the down-regulation of cyclins A and B, resulting in cell cycle arrest at the G2/S-checkpoint in many cells

- figure 1.

Figure 1. Punicalagin-induced cell death and growth inhibition of U87MG glioma cells. (A) Cell viability after 24 or 48 h of incubation with different concentrations of punicalagin was determined using an MTT assay, and the data are presented as the mean±SEM of triplicate experiments. The viability of untreated cells was included as 100% control. A significant difference (*P<0.05) between the experimental group and the control. (B) Cells were treated with different concentrations of punicalagin for 24 h, and the lysates were collected and immunoblotted with antibodies specific for cyclins A, B, and E, Cdk2, p21, p27, and α-tubulin. Protein quantitation data are located in the right panel and were normalized to α-tubulin. (C) Cell cycle distribution from cultures treated with different concentrations of punicalagin for 24 h. The cell cycle assignment was based on the DNA content detected following PI staining using flow cytometry analysis and was representative of more than two experiments.
µg/mL. In addition, the percentage of cells in the sub-G1 phase increased from 3% to 30%; the sub-G1 population represents the cells that are in the late stages of apoptosis. However, cyclin E is a positive regulator of cell cycle progression and is expressed during late G1 phase of the cell cycle; cyclin E mediates the initiation of DNA synthesis by activating cyclin-dependent kinase 2 (Cdk2). Abnormally high expression levels of cyclin E have frequently been found in cancer cells. Up-regulation of cyclin E and p27Kip1 expression was considered to be potentially useful in predicting the prognosis.
of patients with astrocytomas\cite{22}. Transfection of short hairpin RNAs (shRNA) against cyclin E induces apoptosis in cancer cells and abolishes the ability of U-373-MG glioma cells to induce tumor growth in nude mice\cite{23}. These contradictory findings pave the way for additional experiments to elucidate whether cyclin E is specifically expressed in cells undergoing autophagy or apoptosis and to determine a potential role of cyclin E and increased p27 phosphorylation at Thr 198 in autophagy.

Malignant gliomas are resistant to various pro-apoptotic therapies, such as radiotherapy and conventional chemotherapy. In this study, we showed that punicalagin was cytotoxic to human glioma cells by inducing both apoptotic and autophagic cell death. When cells were pretreated with the caspase inhibitor z-DEVD-fmk, only a portion of the observed cell death was prevented, indicating that punicalagin may also activate non-apoptotic cell death. These results point to autophagy or type II cell death as another possible mode of cell death induced by punicalagin. Consistently, incubation of cells with punicalagin resulted in an increase in LC3-I cleavage, and the inhibition of autophagy by chloroquine partly reversed the punicalagin-induced cell death.

The autophagosome fuses with the lysosome to become the autolysosome, in which lysosomal hydrolases degrade the sequestered cellular constituents\cite{12}. Therefore, cells degrade and recycle macronutrients or damaged subcellular organelles through autophagy, which enhances cell survival under a limited supply of nutrients and oxygen. Excessive autophagy, however, may result in autophagic cell death. Whether autophagy represents a mechanism that allows tumor cells to survive from therapy or a mechanism for initiating a non-apoptotic form of programmed cell death is strongly debated\cite{24}. Autophagy may serve as a survival pathway in tumor cells treated with apoptosis activators, and inhibition of autophagy by chloroquine has been used to enhance the ability of either p53 activation or alkylating drug therapies to induce tumor cell death\cite{25}. Chloroquine, a weak base, is concentrated in the acidic lysosomal vesicles and raises the intralysosomal pH, thereby disrupting the function of the lysosomal enzymes required for autophagy. Indeed, chloroquine may improve mid-term survival when used as an adjuvant in combination with conventional therapy for glioblastoma multiforme\cite{26, 27}. However, this study showed that chloroquine alleviated, instead of enhanced, the punicalagin-induced cell death.

Figure 3. Punicalagin induces autophagic cell death in U87MG cells. (A) Cells were pretreated with or without z-DEVD-fmk for 15 min prior to treatment with 10 µg/mL punicalagin for 24 h. Cell viability was determined using an MTT assay, and the viability of the untreated cells was measured as the 100% control. The data are presented as the mean±SEM of triplicate experiments. b, P<0.05 vs the 100% control. e, P<0.05 vs the 100% control and the inhibitor control. (B) Cells were treated with 10 µg/mL punicalagin for 24 h, and the lysates were collected and immunoblotted with antibodies specific for LC3 and α-tubulin. Protein quantitation data are located in the right panel and were normalized to α-tubulin. (C) Cells were transfected with GFP-LC3 or enhanced green fluorescence protein (EGFP) and were then treated with 10 µg/mL punicalagin for 24 h. All images were taken with an Olympic fluorescence microscope (Model IX71) immediately after fixation by paraformaldehyde. Arrows indicate the punctate dots.
death, suggesting punicalagin may initiate autophagic cell death. Consistent with our findings, autophagic cell death can be induced by arsenic trioxide, rapamycin, or cucurmin in malignant glioma cells\footnote{28-31}.

The mTOR kinase has been demonstrated to be a critical regulator of autophagy; activation of mTOR via Akt and
MAPK signaling suppresses autophagy, and the suppression of mTOR via AMPK and p53 signaling promotes autophagy. The role of AMPK in determining autophagy appears to be controversial. Activation of AMPK by metformin or 5′-amino-4-imidazolecarboxamide (AICAR) has been shown to exert cytoprotective effects in cardiomyocytes and neuronal cells[12, 33], and AMPK activation can arrest cell cycle progression and induce cell apoptosis in prostate cancer[34] and a number of chemo-resistant colon cancer cells[35]. Our data suggest that AMPK may mediate multiple mechanisms underlying the in vitro anticancer effects of punicalagin. Treatment of cells with punicalagin resulted in many molecular features of apoptosis, and we demonstrated that although punicalagin can trigger apoptosis, an alternative pathway of cell death, autophagy, is also activated. AMPK has been considered as an universal regulator of autophagy[19]. AMPK can be activated by liver kinase B1 (LKB1) or by various Ca2+ mobilizing agents; active AMPK inhibits the mammalian target of rapamycin complex 1 (mTORC1), resulting in cell autophagy. In this study, we showed that punicalagin activated AMPK and, at the same time, increased the phosphorylation of p27 at Thr 198. Although the detailed mechanism underlying autophagy induction remains unclear, our data agree with the results of Liang et al, who showed that activation of the LKB1-AMPK pathway increases the phosphorylation of p27kip1 at Thr 198, resulting in the stabilization of p27kip1 and subsequently the induction of cell autophagy[19].

In summary, we have identified a novel activity for punicalagin in human glioma cells, namely, the ability to induce apoptosis and autophagy. Therefore, punicalagin may be useful in the development of adjuvant therapies to treat glioma.

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Author contribution
Shyang-guang WANG, Horng-mo LEE, and Yuan-nian HSU designed the experiments; Ming-hung HUANG, Jui-hsiang LI, and Fu-i LAI performed the experiments; Shyang-guang WANG, Horng-mo LEE, and Yuan-nian HSU analyzed the data; Shyang-guang WANG and Horng-mo LEE wrote the manuscript.

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