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Lauryl Gallate Induces Apoptotic Cell Death through Caspase-dependent Pathway in U87 Human Glioblastoma Cells *In Vitro*

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Abstract. *Background/Aim: The treatment of human glioma tumor is still an unmet medical need. Natural products are always promising resources for discovery of anticancer drugs. Lauryl gallate (LG) is one of the derivatives of gallic acid, widely present in plants, that has been shown to induce anticancer activities in many human cancer cell lines; however, it has not been studied in human glioma cell lines. Thus, the effects of LG on human glioblastoma U87 cells were investigated in the present in vitro study. Materials and Methods: Cell morphology and viability were examined by phase-contrast microscopy. Annexin V/Propidium iodide (PI) double staining were performed and assayed by flow cytometry to confirm that viable cell number reduction was due to the induction of apoptosis. Furthermore, U87 cells were exposed to LG in various concentrations and were analyzed by caspase activity assay. To further confirm that LG induced apoptotic cell death, the expression of apoptosis-associated proteins in LG-treated U87 cells was tested by western blot. Results: LG induced morphological changes and decreased viability in U87 cells. Annexin V/PI double staining revealed that LG induced apoptotic cell death in U87 cells in a dose-dependent manner. The increased activities of caspase-2, -3, -8 and -9 demonstrated that LG induced U87 cell apoptosis through a caspase-dependent pathway. In terms of molecular level, LG increased pro-apoptotic proteins Bax and Bak and decreased anti-apoptotic protein Bcl-2 in U87 cells. Furthermore, LG also suppressed the expression of p-Akt, Pak1, Hif-1α and Hif-2α, β-catenin and Tcf-1 in U87 cells. Conclusion: These results suggest that LG induced apoptotic cell death via the caspase-dependent pathway in U87 cells.*

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Glioma is recognized as the most common primary tumor of the central nervous system, comprising about 50~60% of malignant brain tumors (1). Glioblastoma multiforme (GBM) is the most common and most malignant type of glioma in adults (2). Patients with GBM have a median survival time of 1 year, while <5% of patients survive for 5 years, worldwide (3). Currently, the standard treatment of GBM includes surgery, radiation therapy, chemotherapy, or other adjuvant therapy or combinations of radiotherapy and chemotherapy or combined modalities (4-6). The median

survival after brain tumor resection is 8-13 months. However, surgery with radiotherapy and chemotherapy provided significant benefit in median overall survival (21.3 months). Despite many advances in brain tumor therapy, the maximal benefit of treatment is limited and almost all patients relapse (7, 8). Thus, many investigators are focused on discovering new compounds for the treatment of GBM, particularly ingredients derived from natural products.

It is well accepted that compounds capable of inducing cancer cell death by apoptosis would be the best strategy for anticancer therapy. Apoptosis is a normal process of programmed cell death that includes a cascade of events leading to signal transduction (9), cell shrinkage and nuclei condensation (10, 11), and degradation of cellular protein and DNA (12). It is well documented that apoptosis is activated *via* two major routes, the death receptor or extrinsic pathway and the mitochondrial or intrinsic pathway; however, mitochondria play a critical role in the commitment of cells to apoptosis in both pathways (13). So far, many natural compounds have been identified to induce pro-apoptotic effects, and they could be considered as promising candidates for novel cancer therapeutics (14-16).

Lauryl gallate (LG) is a derivative of gallic acid, a natural plant triphenol. LG acts as an antioxidant *via* inhibiting xanthine oxidase and subsequently preventing the generation of superoxide radicals (17), as an anti-bacterial factor through inhibiting the growth of Gram-positive Bacillus subtilis (18), and also as anti-viral against African swine fever virus in Vero cells (19). It is noteworthy that LG has only limited toxicity to normal cells, and thus, it has been used as an antioxidant food additive since long (20). Previous *in vivo* studies have shown that LG not only prevents the formation of dimethylbenzanthracene-induced skin tumours, but also selectively kills tumor cells on established tumours in mice (21).

However, there is no report on the effect of LG in human brain tumor cells. Thus, in the present study, LG was investigated as a potential therapeutic agent for human brain tumor. In particular, the induction of cell apoptosis was examined in U87 human glioblastoma cells treated with LG *in vitro*. The results demonstrated that LG decreased the total viable cell number *via* the induction of apoptotic cell death through mitochondrial pathways and the activation of caspase-3.

Materials and Methods

Chemicals and reagents. LG, propidium iodide (PI), Tris-HCl, trypsin, trypan blue and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). LG was dissolved in DMSO as a stock for further experiments. Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, California, USA).

Cell culture. The U87 human glioblastoma cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and were cultured in DMEM medium supplemented with 10% FBS, 0.1 mg/ml streptomycin, and 100 U/ml penicillin and were incubated at 37˚C in a humidified atmosphere of 5% CO_2 (22).

Cell morphological changes and viability assay. U87 cells (2×10^4 cells/well) were maintained in 12-well plate for 24 h and were treated with LG (0, 0.1, 0.3, 0.5 μM) for 24 and 48 h. After treatment, cells morphological changes evaluated by photographed under contrast-phase microscopy. Cells were harvested and were stained with PI (5 μg/ml) for cell viability by flow cytometry (Becton-Dickinson, San Jose, CA, USA) as previously described (23).

Apoptotic cell death assay. U87 cells (2×10^4 cells/well) were incubated with LG (0, 0.1, 0.25, 0.5, and 1 μM) 24 h. Cells were harvested and were double stained with Annexin V/PI for total apoptotic cell death analysis by flow cytometry as previously described (24).

Measurement of caspase-2,-3,-8, -9 and -12 activities. U87 cells (5×10^5 cells/100 mm-dish) were treated with LG (0, 0.25, 0.5, 1 μM) for 48 h. Cells were collected and resuspended in lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl and 1% Triton X-100). Cell lysate was used for measuring total protein by Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as the standard. Cell lysates (50 μg) were incubated at 37˚C with substrates for each caspase, conjugated to the fluorescent reporter molecule 7-amino-4-trifluoromethyl coumarin (AFC) [Caspase-2 (VDVAD-AFC), Caspase-3 (DEVD-AFC), Caspase-8 (IETD-AFC), Caspase-9 (LEHD-AFC), and Caspase-12 (ATAD-AFC); R&D Systems Minneapolis, MN, USA]. After incubation for 7h, cleavage of the peptide by caspase enzymatic activity released the fluorochrome (excitation/emission=405/510 nm). The level of caspase enzymatic activity was directly proportional to the fluorescence signal detected with a fluorescent microplate reader (Fluoroskan Ascent, Labsystems, Helsinki, Finland) as described previously (27).

Western blotting analysis. U87 cells (5×10^5 cells/100 mm-dish) were incubated with LG at various final concentrations (0, 0.25, 0.5 and 1 μM) for 24 h. Cells were harvested and gently resuspended in lysis buffer (10 mM Tris pH 7.5, 0.5 mM EDTA pH 8.0, 0.5 mM DTT, 0.5% CHAPS, 10% glycerol) supplemented with a cocktail of protease inhibitors (Thermo Fisher Scientific) and were incubated for 30 min on ice. Cell debris were removed by centrifugation at 10,000 g at 4˚C for 20 min. All supernatants were used for measuring total protein by Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as the standard. About 30 μg of each sample were separated by SDS polyacrylamide gel electrophoresis and then electrotransferred onto a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was washed and incubated with blocking buffer (5% BSA, 1X Tris buffered saline, 0.1% Tween 20) for 1 h followed by incubation with primary antibodies against BAX, B-cell lymphoma 2 (BCL2), Bcl-2 antagonist/killer protein (BAK), β-actin, T-cell factor-1 (Tcf-1), Tcf-3, Tcf-4, lymphoid enhancer-binding factor 1 (LEF-1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), hypoxia-inducible factor 1α (HIF-1α), HIF-2α, phosphorylated AKT serine/threonine kinase (p-AKT), phosphorylated p21-activated kinase-1 (p-PAK-1) and β-Catenin (Cell Signaling, Danvers, MA, USA). After washed, the membranes were incubated with HRP-conjugated anti-rabbit IgG

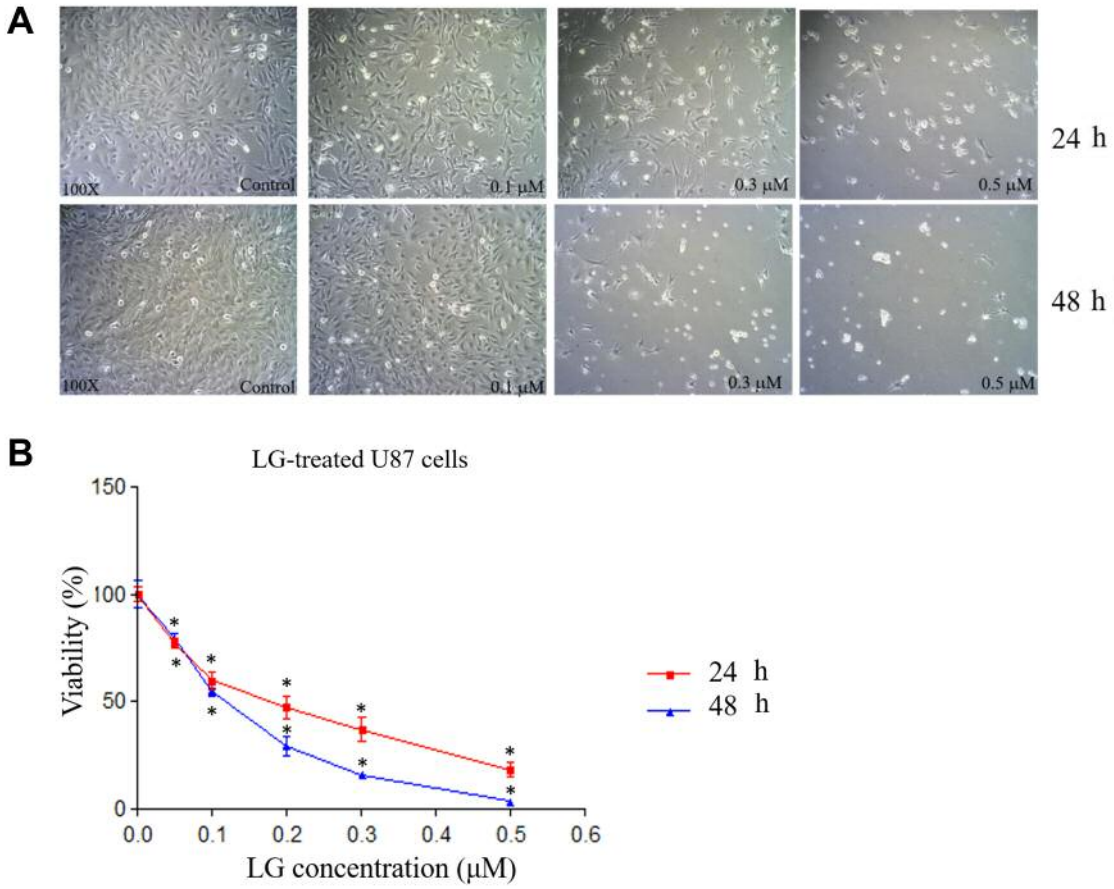

Figure 1. *Lauryl gallate (LG) induced cell morphological changes and decreased cell viability in U87 cells. U87 cells were treated with LG (0, 0.05, 0.1, 0.2, 0.3, 0.5 μM) for 24 and 48 h. A: Cell morphological changes were examined and photographed at 24 and 48 h. B: Total cell viability was measured. *p<0.05, significant difference between LG-treated groups and the control as analyzed by Student's t test.*

secondary antibody (1:10,000) (Cell Signaling). Immunoreactive protein was visualized and detected by ImmobilonTM Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) (23, 24). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for band density quantification.

Immunocytochemistry. U87 cells were fixed with 4% paraformaldehyde in PBS and washed twice with cold PBS. The fixed cells were permeabilized and blocked with 0.1% Triton X-100 and 1% BSA simultaneously for 1 h. Cells were then incubated for 1 h with anti-β-catenin antibody (Cell Signaling) diluted in PBS containing 1% BSA. After incubation with FITC-conjugated secondary antibody (Cell Signaling, MA, USA) at room temperature for 1 h, cells were stained with DAPI (Thermo Fisher Scientific) and mounted over glass slides. Protein expression of b-catenin was evaluated using a confocal laser scanning microscope (Olympus FV1000, Tokyo, Japan).

Statistical analysis. The results were expressed as mean±standard deviation (SD). Figures are representative one of three independent experiments. Statistical analysis was performed by Student's *t*-test with statistical significance of $p<0.05$.

Results

LG induced cell morphological changes and decreased cell viability in U87 cells. U87 cells treated with LG were examined for morphological changes at 24 and 48 h. Results indicated that LG induced cell morphological changes at both treated times based on cell debris and shrinking (Figure 1A). Cell viability was also analyzed, and results showed decreased total viable cell number of U87 cells in a dose-dependent manner (Figure 1B). The IC_{50} value of LG in U87 cells was about 0.2 μM for 24 h treatment.

LG induced apoptotic cell death in U87 cells. To examine how LG induced U87 cell death, U87 cells were treated with LG (0, 0.1, 0.25, 0.5 and 1 μM) for 24 h and then examined by Annexin V/PI double staining followed by flow cytometry. The results indicated that LG induced earlier and late apoptotic cell death (Figure 2A). Furthermore, LG induced apoptotic cell death in U87 cells in a dose-dependent manner

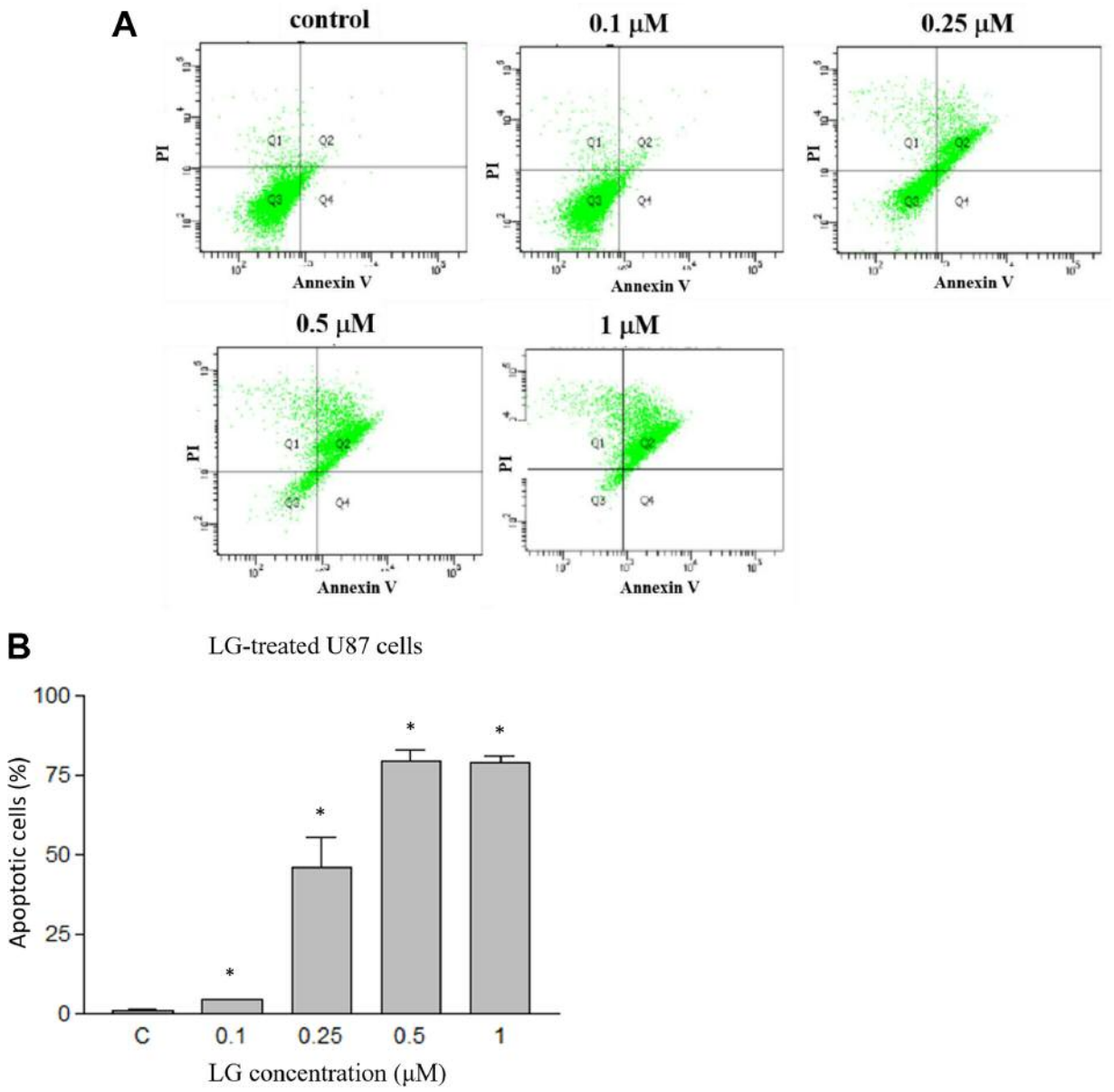

Figure 2. *Lauryl gallate (LG) induced apoptotic cell death in U87 cells. U87 cells were treated with LG (0, 0.05, 0.1, 0.2, 0.25, 0.3, 0.5 and 1.0 μM) for 24 h and apoptotic cell death was determined by Annexin V/PI double staining and analyzed by flow cytometry assay. A: representative profiles; B: calculated percentage of apoptotic cell death. *p<0.05, significant difference between LG-treated groups and the control as analyzed by the Student's t test.*

(Figure 2B). As shown in Figure 2, percentage of Annexin V/PI double positive U87 cells were 1.3±0.4%, 4.6±0.2%, 46.1±16.3%, 79.5±5.9% and 78.9±4.0% after a 24-h treatment with 0, 0.1, 0.25, 0.5 and 1 μM LG, respectively, indicating that LG induced apoptosis in U87 cells.

LG affected the enzymatic activity of Caspase-2, -3, -8, -9 and -12 in U87 cells. To further investigate the involvement of caspases in LG-induced apoptosis, U87 cells were treated with LG (0, 0.1, 0.25, and 1 μM) for 48 h and the activities of caspase-2, -3, -8, 9 and -12 were evaluated for 7 h. Results indicated that LG significantly promoted the activities of caspase-2, -3, -8 and -9 (LG-induced caspase activity: caspase-3>-9>-2>-8) in a dose-dependent manner, but not the activity of caspase-12. Treatment of U87 cells with various concentrations of LG for 48 h resulted in significant increases in the activities of caspase-2, -3, -8, and -9 (Figure 3 and Table I).

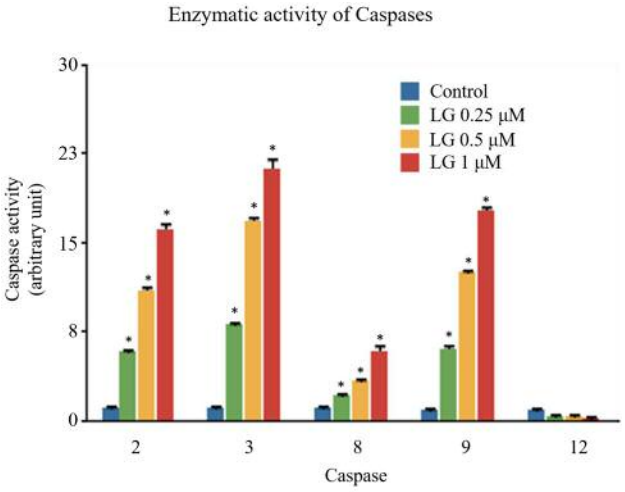

Figure 3. *Lauryl gallate (LG) affects caspase-2, -3, -8, -9 and -12 activities in U87 cells. U87 cells treated with LG (0, 0.1, 0.25, and 1 μM) for 48 h, and the activities of caspase-2, -3, -8, 9 and -12 were measured. *p<0.05, significant difference between LG-treated groups and the control as analyzed by Student's t test.*

LG altered apoptosis-associated protein expression in U87 cells. To further confirm the molecular mechanisms of LG-induced apoptotic cell death in U87 cells, the expression of apoptosis-associated proteins was examined in LG-treated cells. Results indicated that LG significantly increased pro-apoptotic protein expression of BAX and BAK, while decreased the expression of anti-apoptotic protein BCL-2 (Figure 4A). Furthermore, we found that cells treated with LG (only at 1 μM) had significantly decreased expression of HIF-1α and -2α, p-AKT and PAK-1, which are associated with cell survival (Figure 4B). In addition, this study also showed that LG decreased the expression of β-Catenin, TCF-1, TCF-3, TCF-4 and LEF-1 in U87 cells (Figure 4C). The quantitative results of protein expression under LG exposure in U87 cells are shown in Figure 4 D. The expression of β-Catenin in U87 cells was further examined by confocal laser microscopy systems after exposed to LG (0, 0.25, 0.5 and 1 μM) for 24 h. Results confirmed that LG decreased the expression of β-catenin in U87 cells and this effect was dose-dependent (Figure 5).

Discussion

LG has been shown to have antitumor activities *in vitro* and *in vivo* (21, 26) and exert its cytotoxic effects through the induction of apoptotic cell death in many human cancer cell lines (21, 26-27). However, there is no available evidence on LG apoptotic effects on glioma cells *in vitro*. In the present study, we used U87 human glioblastoma cells to demonstrate the potential of LG as an anti-cancer agent *in vitro*.

Table I. *The quantitative results (mean±SD, arbitrary units) of caspase activity in U87 cells after LG treatment.*

	Control	LG 0.25 μM	LG 0.5 μM	LG 1 μM
Caspase-2	1.1±0.1	5.9±0.1*	11.0±0.7*	16.1±0.9*
Caspase-3	1.2±0.2	8.2±0.4*	16.7±0.7*	21.2±1.6*
Caspase-8	1.1±0.1	2.2±0.1*	3.4±0.1*	5.8±1.0*
Caspase-9	1.0±0.0	6.0±0.8*	12.6±0.5*	17.7±0.7*
Caspase-12	0.9±0.1	0.4±0.0	0.4±0.0	0.3±0.0

*$p<0.05$, significant difference between LG-treated groups and the control as analyzed by the Student's *t*-test.

Initially, the cytotoxic effects of LG on U87 cells were examined and LG was found to induce cell morphological changes and decrease the total viable cell number in a dose-dependent manner. In order to investigate whether the LG-induced U87 cell death was due to apoptosis or not, Annexin V/PI double staining, which has generally been used to measure apoptosis of cancer cells (28), was performed. Results indicated that LG reduced the total cell number *via* the induction of apoptotic cell death in U87 cells. These effects were also dose-dependent. Based on our evidence and the literature that suggests the induction of cancer cell apoptosis as a promising strategy for anticancer therapy (29-32), LG is a promising candidate as an anticancer drug for glioma.

It is well known that cancer cell apoptosis can be induced through caspase- dependent and -independent pathways (33, 34). Thus, in order to investigate whether LG induced apoptosis in U87 cells *via* the activation of caspases, the enzymatic activities of caspases -2, -3, -8, -9 and 12 were measured. It was demonstrated that LG increased the activities of all caspases, except casaspe-12, suggesting that LG-induced cell apoptosis in U87 cells is mediated through caspase-dependent pathways. It has previously been shown that the metabolite of cholesterol, pregnenolone, induced caspase-dependent apoptosis in glioma cells *in vitro*, involving Fas-Fas ligand interaction, followed by the activation of caspase-8 and -9 at 24 h, and caspase-3/7 at 48 h (35). The pregnenolone-induced intrinsic apoptosis may be through disruption of lipid rafts (35). Although LG does not share similar structure with pregnenolone, it might influence the properties of lipid rafts followed by induction of intrinsic apoptosis because of its amphipathic structure. However, further investigations should be performed to demonstrate the mechanism.

Moreover, the expression of pro-apoptotic and anti-apoptotic proteins in LG-treated U87 cells was analyzed. It is known that the BCL-2 family proteins are involved in mitochondrial membrane permeability, which is important for the induction of the intrinsic apoptotic pathway, and they can

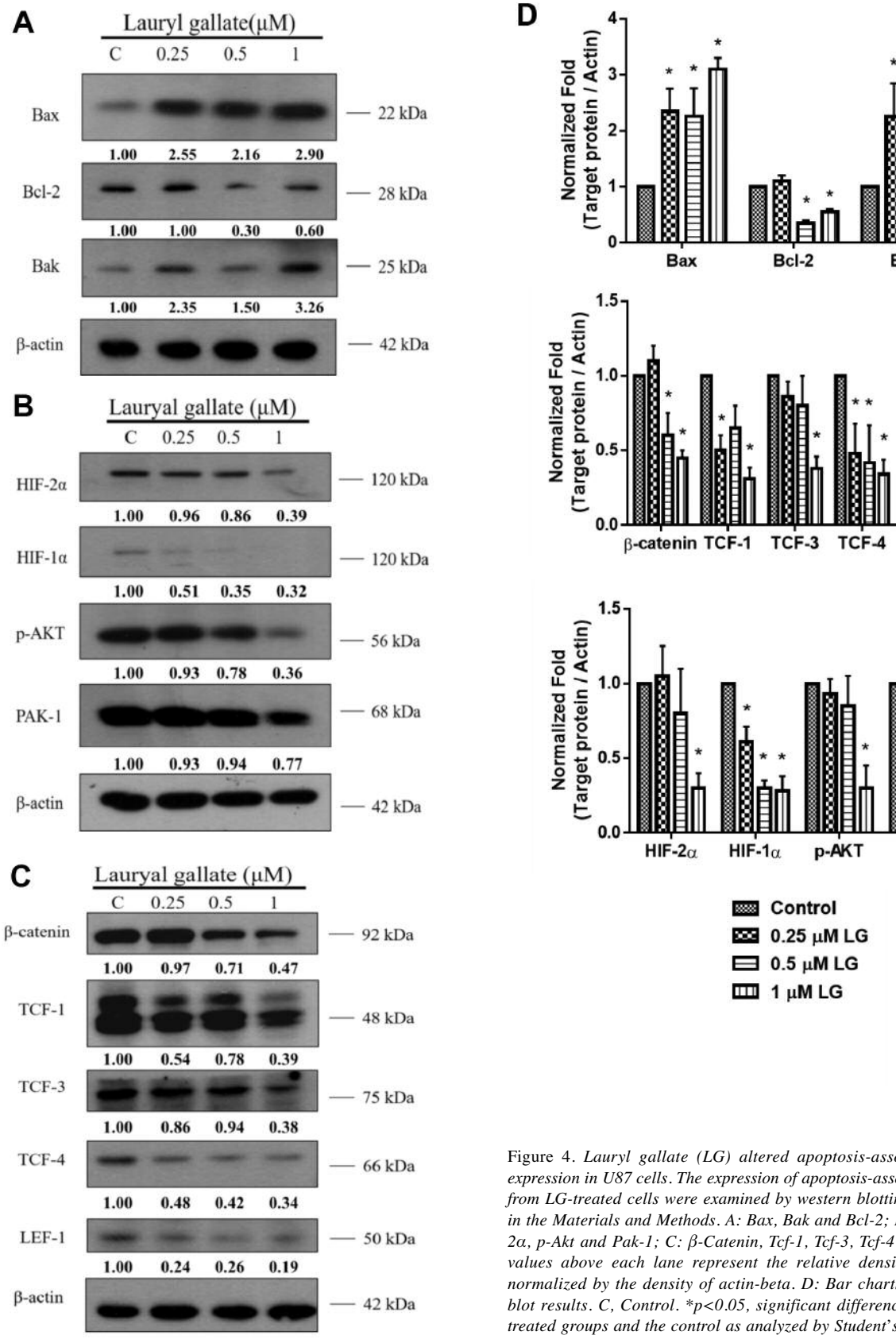

Figure 4. *Lauryl gallate (LG) altered apoptosis-associated protein expression in U87 cells. The expression of apoptosis-associated proteins from LG-treated cells were examined by western blotting as described in the Materials and Methods. A: Bax, Bak and Bcl-2; B: Hif-1α and -2α, p-Akt and Pak-1; C: β-Catenin, Tcf-1, Tcf-3, Tcf-4 and Lef-1. The values above each lane represent the relative density of the band normalized by the density of actin-beta. D: Bar charts show western blot results. C, Control. *p<0.05, significant difference between LG-treated groups and the control as analyzed by Student's t test.*

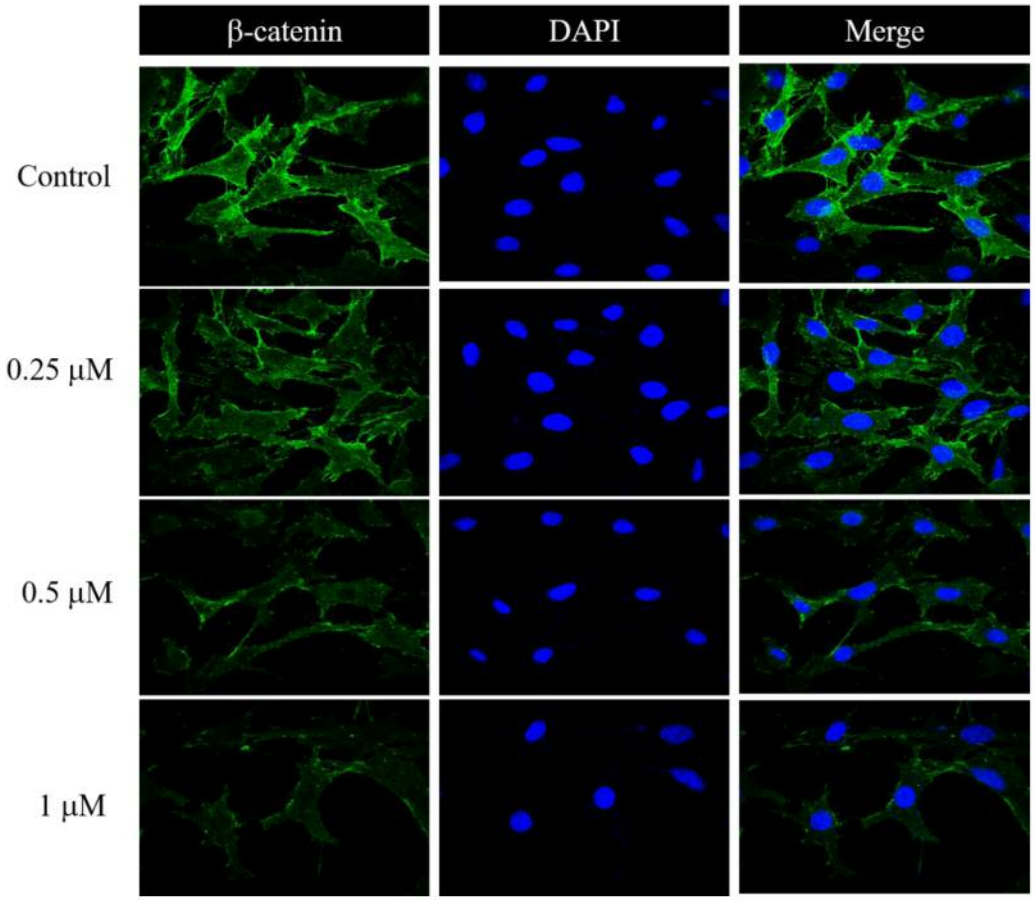

Figure 5. *Lauryl gallate (LG) suppressed the expression of β-catenin in U87 cells. U87 cells treated with 0, 0.25, 0.5 and 1μM for 24 h were examined for the expression of β-catenin. After incubation, cells were collected for staining with anti-β-catenin antibody and were examined and photographed by confocal laser microscopy systems.*

be classified into pro-apoptotic (BAX) and anti-apoptotic (BCL-2) proteins (36, 37). Western blot showed that LG increased the expression of pro-apoptotic proteins BAX and BAK (Figure 4A) and suppressed the level of anti-apoptotic protein such as BCL-2 (Figure 4A) in U87 cells. These results suggested that LG-induced apoptosis of U87 might be through the intrinsic pathway. Furthermore, it has been reported that increased BAX/BCL-2 ratio can affect the mitochondrial membrane potential leading to apoptosis *via* caspase-dependent and/or caspase-independent pathways (38). It has also been documented that the BAX/BCL-2 ratio affects cell-fate determination, and might be involved in the formation of various apoptosis-related diseases, as well (39, 40). Thus, the increase of BAX/BCL-2 ratio induced by LG in U87 cells might alter their mitochondrial membrane potential.

The present results also showed that LG decreased the protein levels of HIF-1α and -2α, p-AKT and PAK-1 in U87 cells (Figure 4B). HIF-1α is a transcription factor and a critical regulator for cellular oxygen balance, since it is also involved in cellular survival during hypoxia (41). Numerous studies have shown that HIF-1α inhibition induces apoptosis in many cancer cell lines, including glioblastoma (42, 43). Furthermore, previous studies have reported that downregulation of MCL-1 and HIF-1α is implicated in curcumin-mediated apoptosis in infantile hemangioma endothelial cells (44). AKT is also involved in cell survival (45, 46); It is activated by phosphorylation (47) and, herein, it was shown that LG inhibited the expression of p-AKT (Figure 4B). Inhibition of AKT phosphorylation may increase susceptibility of cells to apoptosis, which has been previously reported in pancreatic beta-cells (48).

The present results also showed that LG suppressed the expression of PAK1 in U87 cells in a dose-dependent manner. Previous studies on melanoma and gastric cancer have reported that PAK-targeted therapeutics might have anti-tumor effects (49, 50). Currently, many investigators are

focused on identifying selective PAK inhibitors for specific tumors (51). Moreover, previous studies have demonstrated that TCF-1 has higher expression in some cancer cells and suppression of β-Catenin signaling has been shown to suppress the pancreatic tumor growth by disrupting nuclear β-Catenin/TCF-1 complex (52). Herein, it was demonstrated that the expression of β-Catenin and TCF-1, -3 and -4 and LEF-1 was decreased in LG-treated U87 cells. We also used confocal laser microscopy systems to examine the β-Catenin expression of LG-treated cells and results confirmed that LG suppressed the expression of β-Catenin in U87 cells, in a dose-dependent manner (Figure 5).

To our knowledge, this is the first study to show that LG induces apoptotic cell death in U87 glioblastoma cells *in vitro*. The effects of LG were mediated *via* caspase-dependent pathways, as shown by increased expression of pro-apoptotic proteins and decreased expression of anti-apoptotic BCL-2. Thus, our results suggest that LG is a promising candidate for anti-tumor therapy for GMB.

Acknowledgements

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