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Apogossypolone inhibits cell growth by inducing cell cycle arrest in U937 cells

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Abstract. We examined the effect of apogossypolone (ApoG2), a new derivative from gossypol on cell cycle regulation in U937 human leukemic monocyte lymphoma cells *in vitro*. ApoG2 decreased the viability of U937 cells by inducing G1 arrest followed by apoptosis in a dose-dependent manner. The G0/G1 phase of the cell cycle is regulated by cyclin-dependent kinases (Cdk), cyclins and cyclin-dependent kinase inhibitors (Cdki). We show by Western blot analysis, that the ApoG2-induced G1 arrest was mediated through the increased expression of Cdki proteins ($p21^{cip1/waf1}$) with a simultaneous decrease in cdk2, cdk4, Cyclin D1 and Cyclin E expression. The induction of apoptosis after treatment with ApoG2 for 12, 24 and 48 h was demonstrated by flow cytometry analysis. ApoG2 also induced cytochrome c release and activation of caspase-3. To our knowledge, this is the first time that ApoG2 has been reported to potently inhibit the proliferation of human monocytic lymphoma U937 cells through G1 arrest. These findings suggest that ApoG2 may be a potential chemotherapeutic agent for the treatment of cancer.

Introduction

The cell cycle, or cell-division cycle, is the series of events that take place in a eukaryotic cell leading to its replication. Many studies have shown an association of abnormal cell cycle regulation and apoptosis with cancer, in as much as the cell cycle inhibitor and apoptosis-inducing agents are being appreciated as weapons for the management of cancer (1-5). Two key classes of regulatory molecules, cyclins and cyclin-dependent kinases (CDKs), determine a cell's progress through the cell cycle (6). Cyclin D/cdk4 complexes are involved in the regulation of early G1, whereas the Cyclin E/cdk2 complex is required for the completion of G1 and initiation of the S phase (7). Cyclin/cdk complexes can be inhibited by interactions with cyclin-dependent kinase inhibitors (Cdki) such as $p21^{cip1/waf1}$.

In addition to cell cycle inhibition, cancer cells may also be iminated by the induction of apoptosis (8). The development of chemopreventive agents designed to induce cell cycle arrest and apoptosis may be useful in the management and treatment of cancer. Anticancer agents may alter regulation of the cell cycle machinery, resulting in an arrest of cells in different phases of the cell cycle and thereby reducing the growth and proliferation even inducing apoptosis of cancer cells.

Gossypol has been found to have antiproliferative activity against tumor cells, such as human colon carcinoma cells (9), head and neck squamous cell carcinoma cells (10), diffuse large cell lymphoma cells (11) and prostate cancer cells (12). Apogossypolone (ApoG2), a derivative of gossypol, which was found to be a non-peptidic small molecule inhibitor targeting Bcl-2 family proteins, has been synthesized. Recent research focused on ApoG2 showed that it can inhibit the growth of diffuse large cell lymphoma cells, nasopharyngeal carcinoma cells and follicular small cleaved cell lymphoma (13-15). Our studies also showed that it can down-regulate the expression of Bcl-xL and Mcl-1 in U937 cells (16). However, it is not fully known whether ApoG2 affects the proliferation and the cell cycle in U937 cells. Here, we studied the effect on cell cycle of U937 cells and further investigated the molecular mechanism by ApoG2. As a result, we found that ApoG2 can induce G1 arrest in U937 cells. The expression of Cyclin E/CDK2, Cyclin D1/CDK4 and c-myc can be deduced after ApoG2 treatment. Moreover, the $p21^{WAF1}$ was up-regulated. ApoG2 induced caspase-3 activation and cyto-c release from mitochondria in U937 cells.

Materials and methods

Cell line and reagents. U937 cells (human leukemic monocyte lymphoma cell line) were maintained in RPMI-1640

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Abbreviations: ApoG2, apogossypolone; CDKs, cyclin-dependent kinases; Cyto-c, cytochrome c; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PBS, phosphate-buffered saline

Key words: U937, ApoG2, cell cycle arrest, cyclin, cyclin-dependent kinases

(Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% FBS (Gibco Corp., Carlsbad, CA, USA), 1 unit/ml penicillin G and 1 μg/ml streptomycin at 37˚C and 5% CO_2. ApoG2 was dissolved in dimethylsulfoxide (DMSO) to make a stock solution of 20 mM. The primary antibodies used for immunoblots were anti-caspase-3 (sc-7272; Santa Cruz, CA, USA), anti-CDK2 (sc-163; Santa Cruz), anti-cyto-c (sc-13156; Santa Cruz), anti-CDK4 (Cat.2906; Cell Signaling), anti-Cyclin D1 (sc-8396; Santa Cruz), anti-Cyclin E (sc-25303; Santa Cruz), anti-$p21^{CIP1/WAF1}$ (sc-817; Santa Cruz) and anti-c-myc (sc-70468; Santa Cruz).

MTT assay. The *in vitro* cytotoxic effect of ApoG2 toward U937 cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, U937 cells were plated in 96-well tissue culture plates (Costar, Cambridge, USA) at a density of $8x10^4$ cells/ml. Serial dilutions were made from a stock solution of ApoG2 to the desired concentrations. All experimental concentrations were tested in triplicates. Four hours before the desired time-points, 10 μl of MTT (5 mg/ml) was added. Then, after 4-h incubation, optical density values were measured using a spectrophotometric microtiter plate reader (Thermo Multiskan MK3; Thermo Labsystems, Finland) at 570 nm and 630 nm. Percent absorbance relative to control was plotted as a linear function of drug concentration. The 50% inhibitory concentration (IC_{50}) was identified as the concentration of drug required to achieve 50% growth inhibition relative to control populations. Inhibition of cell growth was measured by the percentage of cells that were viable compared to the control (%) = $(OD_C - OD_T)/OD_C x100\%$. OD_T is the OD values of treated samples, and OD_C is the OD values of control samples.

Apoptosis and cell cycle analysis. Flow cytometry analysis was used to assess the cell cycle phase distribution and the percentage of cells having a subdiploid DNA content. Briefly, U937 cells were incubated with 0.1% DMSO or 5, 10 and 20 μM of ApoG2 for 12, 24 or 48 h. Then cells were harvested, washed with PBS and fixed in 70% ice-cold ethanol overnight. The fixed cells were incubated with 5 μg/ml RNase I and 1 μg/ml propidium iodide. The cellular DNA content was determined by a flow cytometer (Beckman Coulter, Fullerton, CA). Apoptotic cells were identified by the sub-G1 phase in the cell cycle distribution.

Immunoblotting analysis of $p21^{CIP1/WAF1}$, c-myc, Cyclin E, Cyclin D1, CDK2 and CDK4. U937 cells were treated with 10 μM of ApoG2 for 0, 12, 24 or 48 h. Whole cell lysates were prepared by adding 2X SDS sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 0.02 mg/ml bromophenol blue, and 5% mercaptoethanol). Equal amounts of protein (50 μg per sample as determined by UV-spectrometry) were electrophoresed on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked for 60 min at room temperature with 5% non-fat dry milk/TBS-Tween-20 and reacted with appropriate antibodies for $p21^{CIP1/WAF1}$, c-myc, Cyclin E and Cyclin D1 (1:1000 dilution in blocking buffer) overnight at 4˚C with gentle rocking. Following incubation with the primary antibody, membranes were washed in TBS-Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000 dilution in blocking buffer) for 1 h at room temperature. Protein was visualized by incubation with enhanced chemiluminescence detection reagents (#7072, Cell Signaling), followed by exposure to X-ray film (Kodak, Rochester, NY). Immunoblot data were quantitated with Quant analysis software (Quantity One; Bio-Rad, Hercules, CA, USA).

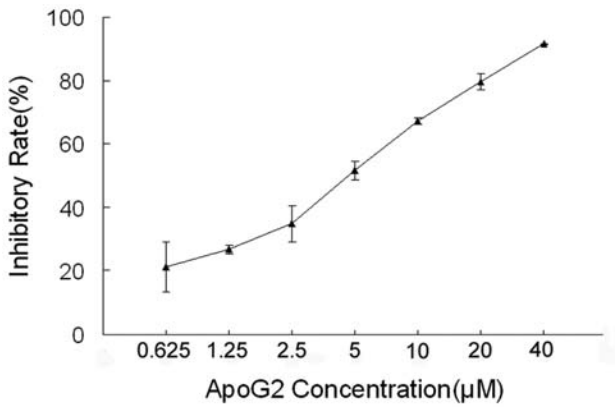

Figure 1. Anti-proliferative effect of ApoG2. Cell survival was determined with MTT assay and the IC_{50} value was calculated as described in the Materials and methods. Each value represents the mean ± SD of three independent experiments.

Detection of cytochrome c release from isolated mitochondria. U937 cells ($3x10^6$ cells) were harvested by centrifugation, washed with PBS, and resuspended in resuspension buffer (30 mM sucrose, 75 mM KCl, 3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 1.5 mM PMSF, 3 μg/ml leupeptin, and 20 μg/ml aprotinin). The resuspended cells were then homogenized using an ultrasounded homogenizer. Resultant homogenates were centrifuged at 3,000 rpm for 5 min to clear nuclear fragments. Cytoplasmic and mitochondrial fractions were further isolated by centrifugation at 15,000 rpm for 20 min at 4˚C. The mitochondria-enriched pellet fraction was then resuspended in resuspension buffer to achieve a protein concentration of 2 μg/μl. The resuspended mitochondria (100 μg per sample) were treated with 0.1% DMSO or 1.0, 10, 25 and 50 μM ApoG2 for 1 h at 37˚C. Following treatment, mitochondria were centrifuged for 20 min at 15,000 rpm at 4˚C. The supernatants, containing released cytochrome c, were removed and subjected to immunoblotting with anti-cyto-c antibody.

Caspase-3 cleavage analysis. Log-phase cell cultures were treated with 0.1% DMSO or 10 μM ApoG2 for 6, 12 and 24 h, harvested by centrifugation. The cell pellets were washed with PBS, and resuspended in 2X SDS sample buffer. Protein (50 μg per sample) was electrophoresed on 12% SDS-PAGE gels, transferred to PVDF membranes, and probed with anti-caspase-3 monoclonal antibody (Santa Cruz).

Statistical analysis. All assays were performed in triplicate. Data are expressed as the mean ± SD. Statistical analyses were performed using ANOVA analysis by SPSS 14.0

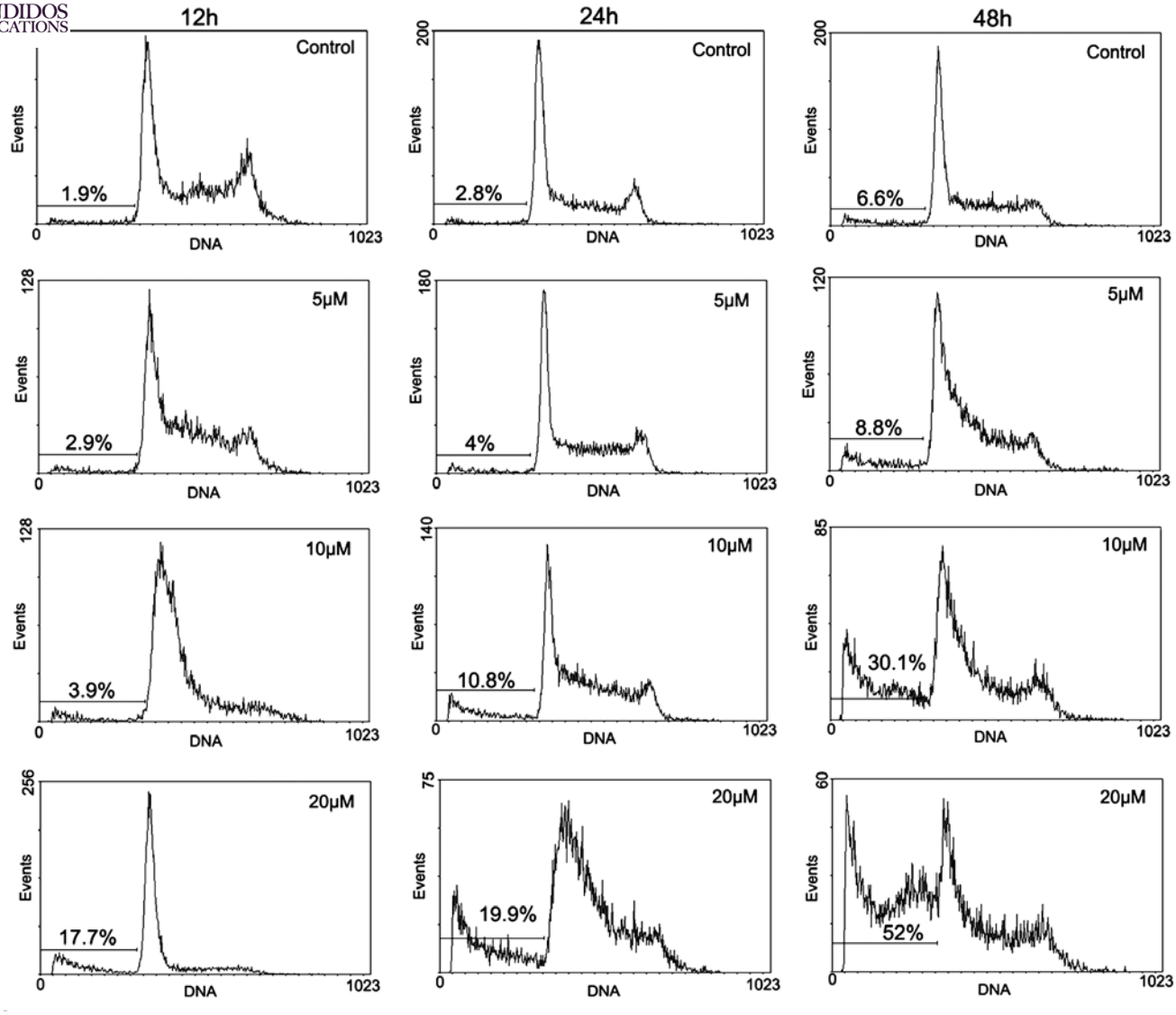

Figure 2. Apoptosis induced by ApoG2. U937 cells were treated with the indicated concentrations of ApoG2 for 12, 24 and 48 h. The percentage of apoptotic cells were analysised by flow cytometry.

software (SPSS Inc., Chicago, IL, USA). A value of $P<0.05$ was considered statistically significant.

Results

Anti-proliferative effect of ApoG2. To investigate whether ApoG2 has an anti-proliferative effect, MTT assay was performed on U937 cells after 72 h of treatment with ApoG2. ApoG2 strongly inhibited the proliferation of U937 cells in a dose-dependent manner, with IC_{50} values of 9.87 μM for 72-h treatment (Fig. 1).

ApoG2 induces apoptosis in U937 cells. Propidium iodide (PI) staining and flow cytometry were used to investigate whether ApoG2 inhibits the growth of U937 cells by initiating the apoptotic pathway. As shown in Fig. 2, U937 cells underwent apoptosis after being exposed to ApoG2 for 12, 24, or 48 h at a range of concentrations (from 5 to 20 μM). The percentage of apoptotic cells in the sub-G1 phase of the cell cycle increased in a time- and dose-dependent manner.

Effect of ApoG2 on cell cycle distribution. To investigate whether ApoG2 had an effect on the cell cycle regulation, we cultured U937 cells with various concentrations (from 5 to 20 μM) for 12 h and analyzed DNA content by flow cytometry. As shown in Fig. 3, exposure of U937 cells to ApoG2 induced a dose-dependent accumulation of G1 phase proportion in U937 cells ($P<0.05$), accompanied by a decrease in the percentage of cells in S phase. Then the cells underwent apoptosis (sub-G1 peak appeared). The ApoG2-induced G1 block peaked at 20 μM, with ~75.8% of the cells in G1 at this time compared with 33.1% in the control.

Regulation of G1 cell cycle regulatory proteins by ApoG2. To determine the molecular mechanisms of ApoG2-mediated G1 arrest, we examined the expression of G1-associated proteins in U937 cells after ApoG2 treatment. As shown in

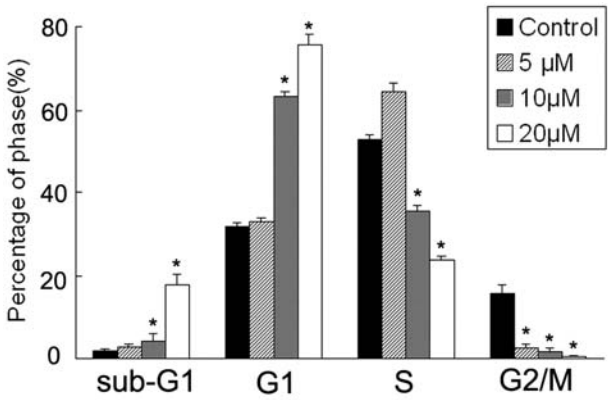

Figure 3. Effect of ApoG2 on cell cycle progression. U937 cells were untreated or treated with 5, 10 and 20 μM ApoG2 for 12 h. After treatment, U937 cells were fixed and stained with propidium iodide. Cell cycle distribution was examined by flow cytometry.

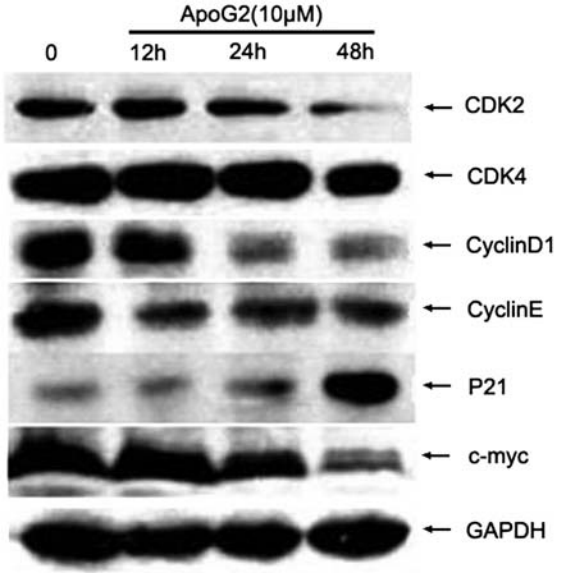

Figure 4. Effect of ApoG2 on the expression of cell cycle regulatory proteins. Cells were treated with the indicated concentrations of ApoG2 for 12 h. After treatment, whole cell protein extracts were prepared as decribed in Materials and methods. Equal amounts of total protein were resolved on SDS-PAGE. Western blot analysis was performed using specific antibodies against the indicated proteins. Blots were reprobed with GAPDH to normalize each lane for protein content.

Fig. 4, after 12 h of ApoG2 treatment, U937 cells showed a significant increase in $p21^{CIP1/WAF1}$ protein levels, which gradually increased up to 48 h. In contrast, levels of Cyclin E, Cyclin D1, CDK2, CDK4 and c-myc were markedly decreased in ApoG2-treated U937 cells in comparison with controls. These results indicate that the increase in $p21^{CIP1/WAF1}$ and decrease in Cyclin E, Cyclin D1, CDK2, CDK4 and c-myc might be involved in ApoG2-mediated G1 arrest.

ApoG2 promotes cytochrome c release in isolated mitochondria. Demonstration that ApoG2 acts directly on the mitochondria would support the hypothesis that a Bcl-2 family member(s) is a direct intracellular target of ApoG2.

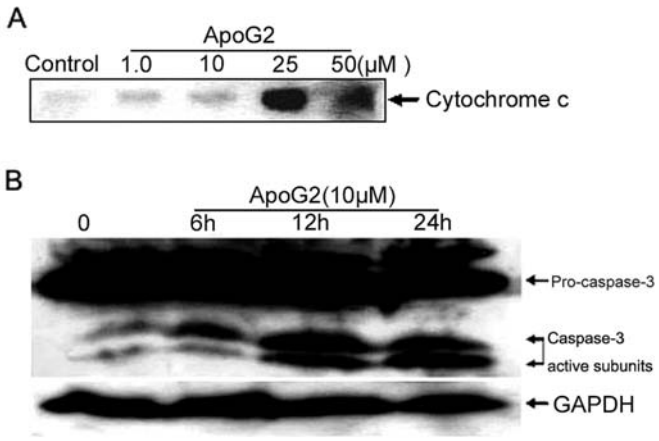

Figure 5. ApoG2 promotes cytochrome c release in isolated mitochondria and determination of cytosolic caspase-3 levels. Mitochondria were isolated from U937 cells as described in Materials and methods. The mitochondria (100 μM per sample) were incubated for 1 h at 37˚C in the presence of 0.1% DMSO, or the indicated concentrations of ApoG2. Following incubation, mitochondria were pelleted, and the corresponding supernatant proteins, containing released cytochrome c, were electrophoresed on a 15% SDS-PAGE gel, and probed with anti-cytochrome c (A). U937 cells were treated with 10 μM ApoG2 for 6, 12 and 24 h. The total cell lysates for caspase-3 was processed for Western blot analysis as described in Materials and methods. Protein (50 μg) for each sample was resolved by 12% SDS-PAGE and Western blot analysis was performed. GAPDH was used as an internal control (B).

Here we examined the impact of ApoG2 on purified mitochondria isolated from U937 cells. Mitochondria were isolated from U937 cells as described in Materials and methods. After 1 h of ApoG2 treatment, supernatants were subjected to immunoblotting to detect released cytochrome c. As shown in Fig. 5A, a dose-dependent release of cytochrome c was observed upon treatment with 1.0-50 μM of ApoG2. Treatment with 0.1% DMSO did not lead to a substantial release of cytochrome c. Amido black staining showed equivalent loading in all lanes (data not shown). Cytochrome c was observed to be released from the mitochondria at sufficient concentrations for ApoG2 to induce cell death. These data indicated that ApoG2 acts directly on the mitochondria in intact U937 cells.

ApoG2 promotes caspase-3 activation in U937 cells. The activation of caspase-3 is known to occur downstream of the mitochondrial cytochrome c release during drug-induced apoptosis (17,18). To verify that ApoG2 could promote apoptosis signaling in U937 cells, we investigated activation of the major cellular executioner caspase, caspase-3. Caspase-3 is synthesized as a 32-kDa zymogen form, which is cleaved during apoptosis to yield active subunits of 17 and 10 kDa. Immunoblotting revealed that ApoG2 efficiently induced caspase-3 processing in U937 cells (Fig. 5B).

Discussion

ApoG2, a new derivative from gossypol, has been previously shown to have anti-tumor activity in several cancers (13-15). However, neither the effects on cell cycle nor further

r mechanism analysis by ApoG2 have not been. In this study, we selected human monocyte lymphoma U937 cell line to study how ApoG2 affected the cell cycle and the possible related molecular mechanisms. The antiproliferative and apoptosis-inducing effect of ApoG2 on U937 cells was evident from the results. The percentage of viable cells was significantly decreased by MTT measurement, thereby showing the antiproliferative activity of ApoG2.

It has been shown that the arrest of cells at the checkpoints of the cell cycle occurs as an event preceding the detection of apoptotic cells (3). Our data from cell cycle analysis showed that there was a prominent increase in the G0/G1 DNA content upon ApoG2 treatment. The increase of DNA content indicated the retardation of cell cycle, which might have taken place during the G1/S transition phase. This is a significant observation because the regulation of cell cycle and apoptosis machinery play an important role in the growth and development of cancer (19-21). Here, we performed further studies to elucidate the mechanism of cell cycle arrest. We found that the expression of $p21^{CIP1/WAF1}$ was increased during the G1 arrest. Cyclin-dependent kinases (Cdks), the heart of the eukaryotic cell cycle engine, are a family of serine/theronine protein kinases (22), and the activity of Cdks is regulated by $p21^{CIP1/WAF1}$. Since $p21^{CIP1/WAF1}$ is also regarded as universal inhibitors of cyclin-cdk complexes, we examined whether ApoG2 suppressed the expression of cyclins and cdks during the G1 phase of the cell cycle. It has been well documented that Cdk4 and Cdk6 are activated in association with D-type cyclin in the mid G1 phase (23), and Cdk2 is associated with Cyclin E in the late G1 phase and its activity is rate-limiting for progression from the G1 to the S phase (24,25). Moreover, inhibition of cell cycle progression might be associated with an altered expression of cell cycle relevant regulator, including c-myc (26). The present study showed that the reduced levels of these G1-associated cyclins may facilitate blockade of the cell cycle in mid G1 and G1/S in ApoG2-treated cells. Accumulated $p21^{CIP1/WAF1}$ protein may bind to and inhibit the kinase activity of Cdk2 and Cdk4, therefore, preventing cells from entering the S phase and subsequently arresting the cell cycle in the G1 phase.

We also found that ApoG2 induces apoptosis in U937 cells. The mechanism for triggering apoptosis upon cytotoxic therapy may differ for individual stimuli, however, and damage to DNA or to other key signaling molecules appears to be a common inciting event (27,28). A family of cysteine proteases, known as caspases, plays a pivotal role in the execution phase of apoptosis (26). Caspase-3 determines the nuclear alterations of apoptosis. Once activated, caspase-3 rapidly induces the cleavage of PARP, which in turn leads to degradation of DNA into nucleosomal fragments. Activation of the caspase cascades is the major mechanism, which promotes apoptosis in response to death-inducing signals from cell surface receptors, mitochondrial, or endoplasmic reticulum stress (27,29). Our results showed that ApoG2 can stimulate the isolated mitochondria to release cytochrome c and caspase-3 cleavage in U937 cells. These observations clearly indicate that the mitochondrial-mediated activation of caspase-3 cascades is involved in ApoG2-induced apoptosis.

Summarizing the results, we were able to prove that ApoG2 inhibited the proliferation of U937 cells and induced G1 cell cycle arrest, and the subsequent apoptotic cascade. Furthermore, ApoG2 induced cytochrome c release from mitochondria to cytosol, and triggered activation of the caspase-3 cascade, suggesting that ApoG2-induced apoptosis is via a mitochondrial pathway, thereby proving the anticancer property of the drug.

Acknowledgements

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