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Endothelial-monocyte-activating polypeptide II induces rat C6 glioma cell apoptosis via the mitochondrial pathway

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ABSTRACT

The present study was performed to examine whether Endothelial-monocyte-activating polypeptide II (EMAP II) could inhibit glioma growth by inducing rat brain glioma C6 cells apoptosis. The results revealed that the EMAP II decreased cell viability of rat C6 glioma cells in a time-dependent manner. Apoptotic proportion was increased gradually after EMAP II. EMAP II induced the decrease in mitochondrial membrane potential and the release of cytochrome *c* into the cytosol, followed by activation of caspase-9 and caspase-3. Meanwhile, EMAP II-induced apoptosis was accompanied by an increase of reactive oxygen species (ROS). The significant up-regulation in the expressions of Bax and Apaf-1 as well as down-regulation in the expression of Bcl-2 was observed. The time course change of ROS was prior to the changes of above investigated indexes. All of these results strongly suggest that EMAP II could induce rat C6 glioma cells apoptosis via the mitochondrial pathway, and ROS, Bax/Bcl-2 might be involved in this processing.

1. Introduction

Brain glioma is the most common primary intracranial tumor with high recurrence after surgery, and post-operative chemotherapy is currently the main method for prolonging the lifespan of patients [1]. However, the existence of blood-tumor barrier (BTB) in tumor tissue limits the delivery of anti-cancer drugs to brain tumor tissue [2]. Selective opening of the BTB is a crucial event to allowing the entry of anticancer agents into the brain, which is necessary for effective chemotherapy of brain tumors. If we can find an agent, which can not only open the BTB selectively, but also has anti-tumor effect, the chemotherapy efficacy of glioma will be greatly improved.

Endothelial-monocyte-activating polypeptide (EMAP) II is a proinflammatory cytokine originally isolated from the supernatants of methylcholanthrene A-induced fibrosarcoma cells [3]. Studies have shown that EMAP II is capable to inhibit growth, proliferation and spread of multiple primary and metastatic tumors and has been extensively applied to the researches of anti-tumor therapy [4]. With regard to the anti-tumor effect of EMAP II, the researchers found that EMAP II could induce apoptosis of endothelial cells and inhibit tumor angiogenesis [5,6]. These findings suggest that anti-angiogenic functions might be one possible mechanism of EMAP II in vivo antitumor activity.

Recently, our group has demonstrated that EMAP II can increase the BTB permeability through the paracellular and transcellular pathway and the possible mechanism is associated with RhoA/ROCK and PKA signaling pathways [7–9]. Meanwhile, during the process of our preliminary study, we occasionally found that EMAP II could directly inhibit the growth of rat C6 glioma cells. These studies indicated that EMAP II has dual role of opening BTB and anti-tumor. But the exact mechanism of EMAP II inhibiting the growth of C6 cells is unclear. Therefore, in present study, we attempt to investigate the effect and the possible mechanism of EMAP II inhibiting growth on C6 glioma cells.

2. Materials and methods

2.1. Antibodies and chemicals

EMAP II was purchased from PeproTech (Rehovot, Israel). Antibodies against protease activating factor-1 (Apaf-1), cytochrome *c*, cleaved caspase-3, cleaved caspase-9, Bcl-2 and Bax were obtained

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from Cell Signaling Technology (Beverly, MA). Antibodies against β-tubulin and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Other chemicals were of analytical grade and purchased from Sigma–Aldrich.

2.2. Cell culture and cell viability assay

Rat C6 glioma cells were provided by the Department of Neurosurgery, Shengjing Hospital of China Medical University. Cells were cultured in high glucose Dulbecco's modified Eagle medium (DMEM; Sigma–Aldrich) with 10% fetal bovine serum (Gibco) at 37 °C and 5% CO2.

Cell viability was determined by using the conversion of MTT to formazan via mitochondrial oxidation. Rat C6 glioma cells were seeded in 96-well plates with an average of 2×10^3 cells/well. Cells were incubated overnight and then treated in quintuplicate with 0.005, 0.05, 0.5 and 5 nM EMAP II for 0, 0.5, 1, 3, 6, 12, 24, 48, and 72 h. After treatment with EMAP II, the medium was replaced with 0.5 mg/mL MTT medium and incubated for 4 h. The MTT solution was removed from the wells, and the formazan crystals were dissolved in DMSO. Finally, the optical density was read by a microplate reader (Molecular Devices, CA) at a wavelength of 490 nm. All experiments were repeated at least three times.

2.3. Annexin-V-FITC/Propidium iodide double staining assay

Annexin-V-FITC/PI staining was carried out according to the manufacturer's protocol (Annexin-V-FITC kit, Sigma). The rat C6 glioma cells were exposed to EMAP II for 0, 12, 24, 48, and 72 h and then harvested and resuspended in Annexin-V binding buffer. Then 5 μL of Annexin V-FITC and 10 μL of PI were added, and the cells were incubated for 10 min at room temperature in the dark. The cells were analyzed immediately after staining by using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, U.S.A.) and ModFit LT software (Verity Software, Topsham, ME, U.S.A.). For each measurement, at least 20,000 cells were counted.

2.4. Hoechst DNA staining and measurement of mitochondrial membrane potential (MMP, $\triangle\psi m$)

Nuclear changes that are characteristic of apoptosis were visualized by Hoechst DNA staining. Briefly, rat C6 glioma cells were seeded at 2×10^5 cells/well into 6-well plate containing sterile coverslips. Cells were cultured for 12 h to allow adherence to the coverslips, and then exposed to EMAP II. Coverslips were washed with PBS containing 5% heat-inactivated FBS and incubated with 100 μg/mL Hoechst 33342 trihydrochloride dye in PBS for 10 min at room temperature in the dark. Finally, coverslips were washed with PBS containing 5% FBS, and then photographed by using a fluorescence microscope (Olympus, Japan).

The mitochondrial probe JC-1 (Molecular Probes, Eugene, USA) was used to detect MMP changes. Briefly, after treatment with EMAP II, rat C6 glioma cells were harvested and washed in PBS. After that, the cells were incubated with JC-1 dye (2.5 μg/mL) at 37 °C in a 5% CO2 incubator for 20 min. The cells were then washed, resuspended in PBS, and analyzed by flow cytometry.

2.5. Cytochrome c release assay

The release of cytochrome *c* from mitochondria was assayed as described previously [10]. Briefly, the cells were treated with and without EMAP II respectively, harvested, and resuspended in hypotonic buffer. Cells were homogenized and cytosolic fractions were isolated by differential centrifugation. Protein from the cytosolic fractions of each sample was analyzed by immunoblotting by using an anticytochrome *c* antibody (diluted 1:300).

2.6. Caspase-3 and -9 activity assay

Rat C6 glioma cells were treated with EMAP II, and then the cells were extracted with extraction buffer at 4 °C for 15 min. The soluble extracts were collected by centrifugation at 14,000 g and 4 °C for 15 min and stored at −80 °C until assayed. 10 μL of cell extract (20 μg of protein) was added to 100 μL reaction mixture containing either 12 μM Ac-DEVD-pNA or Ac-LEHD-Pna (BIOMOL Research Laboratories) as the substrates for caspase-3 and caspase-9, respectively, in a 96-well plate. After incubation for 2 h at room temperature, the amount of caspase-mediated *p*-nitroaniline-derived substrate cleavage was determined by using a microplate reader at 405 nm.

2.7. Measurement of reactive oxygen species (ROS)

2′,7′-Dichlorofluorescein diacetate (DCF-DA, Invitrogen) is a fluorogenic freely permeable tracer specific for ROS assessment. It can be deacetylated by intracellular esterase to the nonfluorescent 2′,7′-dichlorofluorescein, which is oxidized by ROS to the fluorescent compound 2′,7′-dichloroflorescein. Thus, the fluorescence intensity of 2′,7′-dichloroflorescein is proportional to the amount of ROS produced by the cells. Approximately 1×10^6 cells/well of C6 glioma cells were plated in a six-well plate. After treatment, cells were incubated with 10 μM DCF-DA for 30 min at 37 °C in the dark. The cells were harvested and washed with PBS three times, and the mean fluorescence intensity (MFI) was determined by flow cytometry.

2.8. Western blot

Total protein from cells was extracted in lysis buffer (Pierce, Rockford, IL, USA) and quantified by using the BCA method. Equal amounts of protein (20–40 μg) were separated by SDS-PAGE and processed for immunoblotting with antibodies for cleaved caspase-3, cleaved caspase-9, cytochrome *c*, Bcl-2, Bax, Apaf-1, β-tubulin and GAPDH (diluted 1:300; 1:300; 1:500; 1:250; 1:500; 1:250; 1:500; 1:500, respectively). All the protein bands were scanned and integrated density values (IDVs) were quantified by Fluor Chen 2.0 software and normalized to that of β-tubulin or GAPDH.

2.9. Statistical analysis

The data are presented as the mean ± SD. Student's t-test was used to determine the significant difference between two groups. One-way analyses of variance (ANOVA) and post hoc comparisons (Bonferroni test) were used to determine the significant difference among multiple groups. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of EMAP II on cell viability in rats C6 glioma cells

The effects of EMAP II on the viability of C6 cells were evaluated at 0, 0.5, 1, 3, 6, 12, 24, 48 and 72 h at various concentration levels (0.005, 0.05, 0.5 and 5 nM) of EMAP II. As shown in Fig. 1A, the cell viabilities of C6 were inhibited by EMAP II in time- and dose-dependent patterns. Compared with the EMAP II 0 h group, the cell viability was decreased at 0.5 h with the treatments of 0.05, 0.5 and 5 nM EMAP II, and there was no obvious difference among three groups. Meanwhile, 0.05 nM was the optimum concentration

of EMAP II opening the BTB, thus 0.05 nM was selected as the optimum concentration in the following experiments.

3.2. Effects of EMAP II on the apoptotic proportion and morphological features of rat C6 glioma cells

The Annexin-V-FITC/PI double staining and Hoechst DNA staining assays were used to test the apoptotic proportion. The result showed that EMAP II increased the apoptotic number of C6 cells in a time-dependent manner (Fig. 1B). To further observe the morphologic characteristics of apoptosis, rat C6 cells were exposed to EMAP II and stained with Hoechst 33342. Cells of EMAP II 0 h group showed an even distribution of the stain and round homogeneous nuclei. Apoptotic cells increased gradually in a time-dependent manner and displayed typical changes, including bright stained and condensed or fragmented nuclei (Fig. 1C).

3.3. Effect of EMAP II on MMP and release of cytochrome c of rat C6 glioma cells

The mitochondrial probe JC-1 was used to detect △ψm changes. As is shown in Fig. 1D, EMAP II caused the decrease of △ψm in a time-dependent manner. Compared with EMAP II 0 h, the percentage of cells with a loss △ψm was significantly increased after EMAP II treatment and it continually increased to reach 76.76% at 24 h.

In EMAP II 0 h group, cytochrome *c* was mainly expressed in mitochondria, and only slightly expressed in cytoplasm. After EMAP II treatment, the release of cytochrome *c* from mitochondria

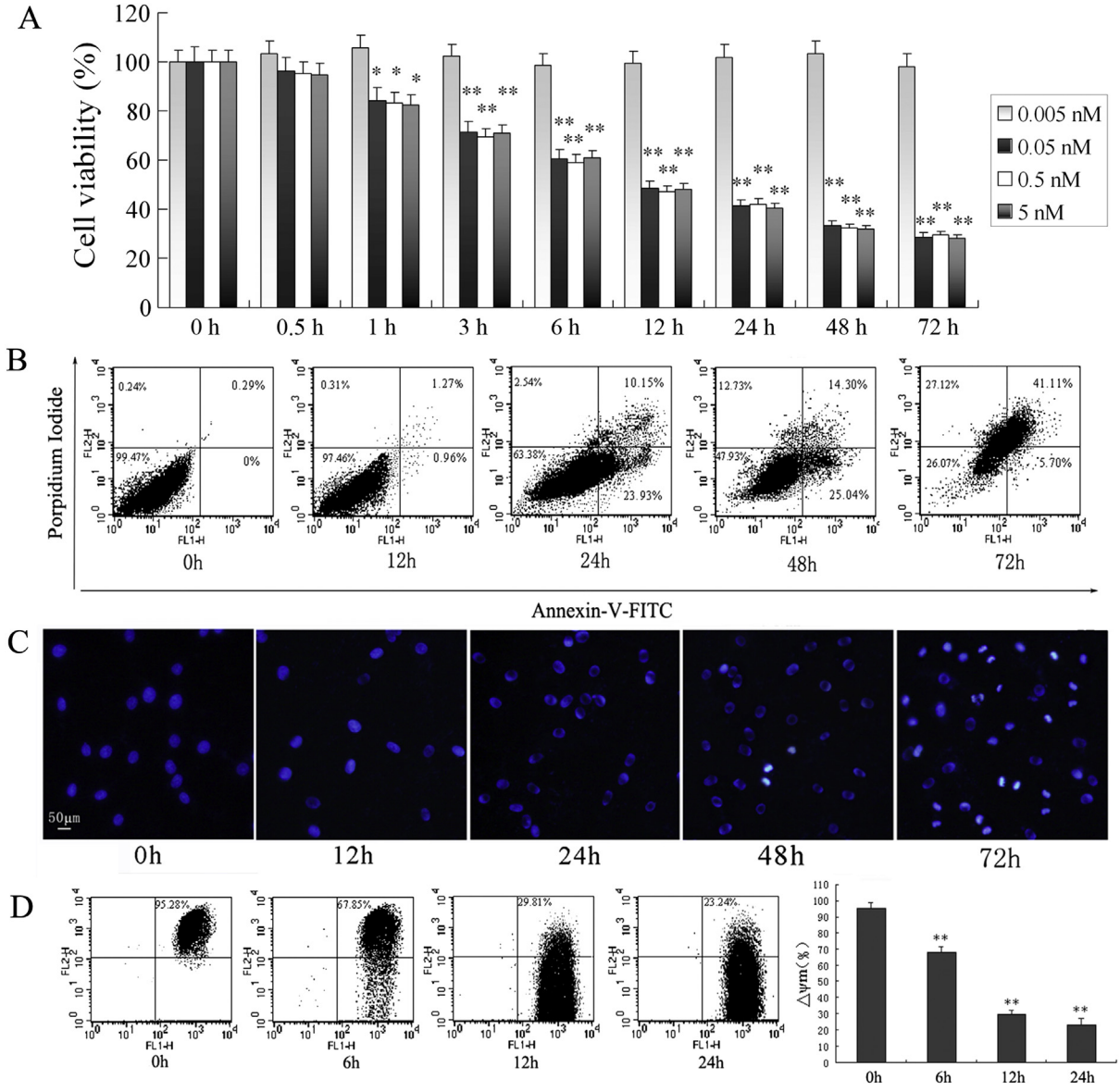

Fig. 1. Effects of EMAP II on rat C6 glioma cells. (A) Effect of EMAP II on the cell viability of C6 glioma cells after treatment with 0.005 nM, 0.05 nM, 0.5 nM and 5 nM EMAP II for 0.5, 1, 3, 6, 12, 24, 48 and 72 h. (B) Apoptosis analysis in rat C6 glioma cells was assessed by Annexin V/PI double staining. (C) EMAP II-mediated apoptosis-related morphological changes were examined by Hoechst 33324 staining. Scale bar = 50 μm. (D) Effect of EMAP II on MMP of rat C6 glioma cells. Quantification of MMP expressed as a ratio of JC-1 aggregate to monomer fluorescence. Values represent the means ± SD (n = 6, each group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. EMAP II 0 h group.

to cytoplasm was increased by 6 h. Thereafter, it was persistently elevated to 72 h (Fig. 2A,B).

3.4. Effects of EMAP II on the activity and the protein expression of caspase-3 and caspase-9 in rat C6 glioma cells

Compared with EMAP II 0 h group, the activity of caspase-9 and caspase-3 significantly increased, and they reached maximum at 12 and 24 h, respectively (Fig. 2B). As is shown in Fig. 2C, the protein expression levels of cleaved caspase-9 and cleaved caspase-3 were also enhanced after treatment with EMAP II. Meanwhile, the changing trends of cleaved caspase-9 and cleaved caspase-3 in the protein expression were consistent with that of their corresponding activity. Combining the result of MMP and that of cytochrome *c*, we considered that the mitochondrial pathway might play an important role in EMAP II-induced apoptosis of rat C6 glioma cells.

3.5. Effect of EMAP II on the production of ROS in rat C6 glioma cells

The intracellular ROS level was analyzed by flow cytometry after incubation with DCF-DA in cells. Compared with EMAP II 0 h group, the cells being stained for ROS were significantly increased after EMAP II treatment. The level of ROS reached its peak at 1 h and then decreased gradually (Fig. 3A).

3.6. Effects of EMAP II on protein expression levels of Bcl-2, Bax and Apaf-1 in rat C6 glioma cells

The protein expression levels of antiapoptotic protein Bcl-2 and pro-apoptotic protein Bax were measured by western blot assay. In EMAP II 0 h group, Bcl-2 presented high expression and only a slight expression of Bax in rat C6 glioma cells. After EMAP II treatment, Bcl-2 was down-regulated and Bax was up-regulated in a time-dependent manner (Fig. 3B).

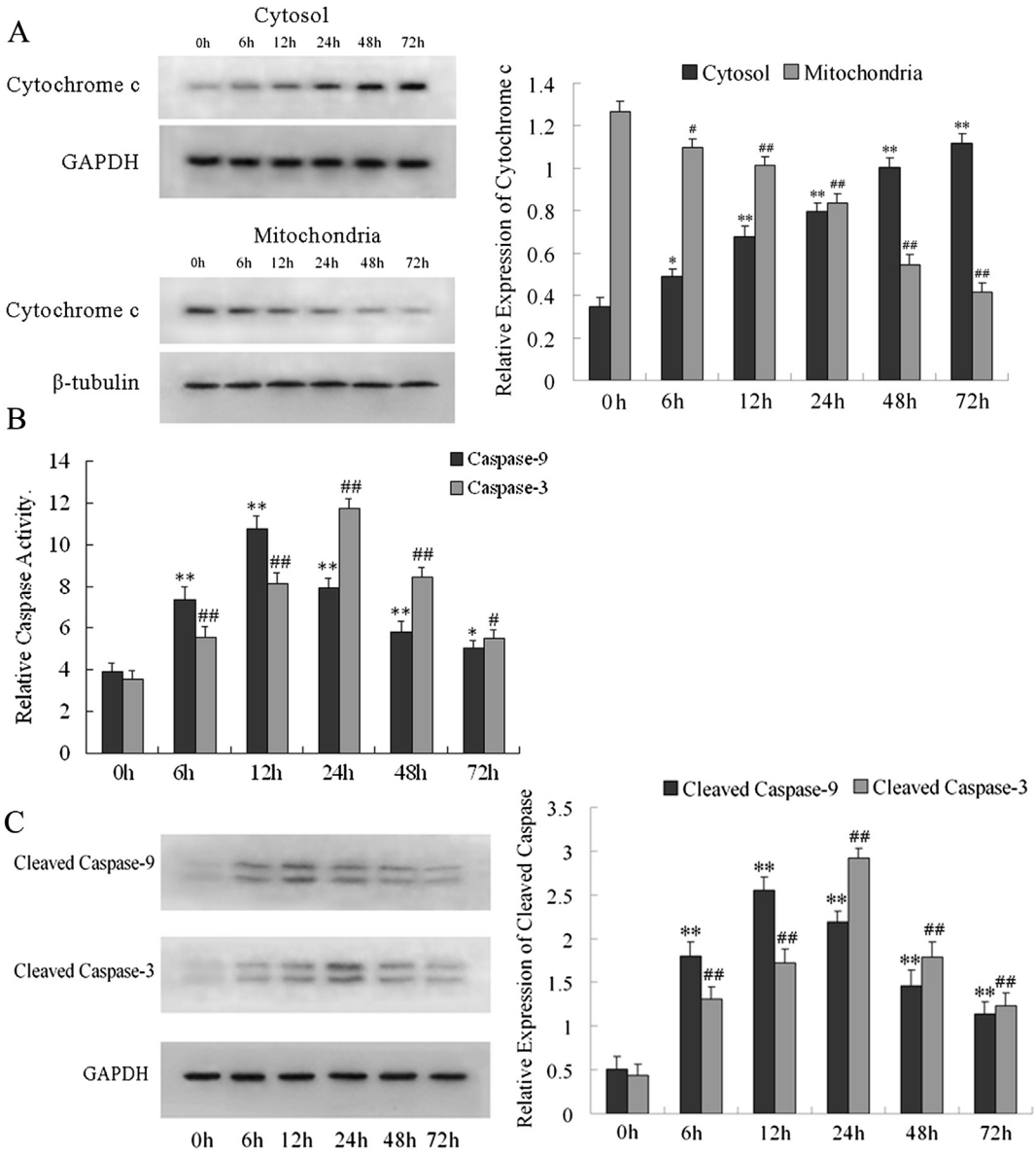

Fig. 2. (A) Effect of EMAP II on cytochrome *c* release of rat C6 glioma cells. (B) Effect of EMAP II on the activities of caspase-3 and caspase-9 in rat C6 glioma cells. The results are expressed in terms of arbitrary fluorescence units per mg protein. (C) Western blot assay was used to detect the cleaved forms of caspase-3 and caspase-9. Values represent the means ± SD (n = 8, each group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. EMAP II 0 h group of cytosol and caspase-9; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. EMAP II 0 h group of mitochondria and caspase-3.

It is well-known that Apaf-1 is involved in apoptosome formation, leading to poptosis. To confirm the further effect of EMAP II on Apaf-1, we detect its protein expression level. The result showed that the protein expression level of Apaf-1 was low expressed in rat C6 glioma cells. After EMAP II treatment, Apaf-1 was increased in a time-dependent manner (Fig. 3C).

4. Discussion

This study showed that the cell viability of C6 glioma cells was inhibited by EMAP II (0.005 nM–5 nM) in a time and dose-dependent manner. Meanwhile, we found that the apoptotic proportion of cells was increased and the morphological changes were induced by EMAP II. Those proved that EMAP II inhibited the cell viability of C6 glioma cells by inducing apoptosis. After that, we further demonstrated that EMAP II induced a significant decease in MMP and the release of cytochrome *c* into the cytosol, followed by activation of caspase-9 and caspase-3. EMAP II caused an increase of intracellular ROS level. At the same time, the increasing Bax expression induced by EMAP II as well as decreasing Bcl-2 expression was observed. The time course change of ROS was prior to the changes of above observed indexes. These results indicated that a ROS-mediated mitochondrial pathway was involved in the process of EMAP II-induced apoptosis of rat C6 glioma cells.

EMAP II has been recognized as an angiostatic mediator that suppresses neovascularization. EMAP II induced endothelial cell apoptosis, inhibited tumor vessel formation, suppressed primary and metastatic tumor growth [11,12] and was found to reduce tumor cell proliferation indices within the neoplastic tissue [13]. In the previous study, we found that EMAP II (0.05 nM) could markedly decrease the viability of C6 glioma cells during the process of researching whether the possible mechanisms of 0.05 nM EMAPII could increase the BTB permeability. Based on these observations, we firstly tested the effect of different doses (0.005 nM–5 nM) of EMAPII on the viability of C6 glioma cells by MTT assay. The result demonstrates that (0.05 nM–5 nM) EMAP II can inhibit the viability of rat C6 glioma cells. Then, we studied the possible mechanism of EMAP II inhibiting the viability of rat C6 glioma cells.

Apoptosis is an important mechanism of antitumor drug induced cell killing and susceptibility to apoptosis of tumor cells is an important determinant of chemotherapy efficacy [14]. Hence, this study verified whether EMAP II inhibits rat C6 glioma cells proliferation by inducing cell apoptosis. We quantified cell apoptosis by flow cytometry and Hoechst DNA staining and found that the proportion of apoptotic cells was increased with the incubation time of increasing EMAP II. Meanwhile, a series of

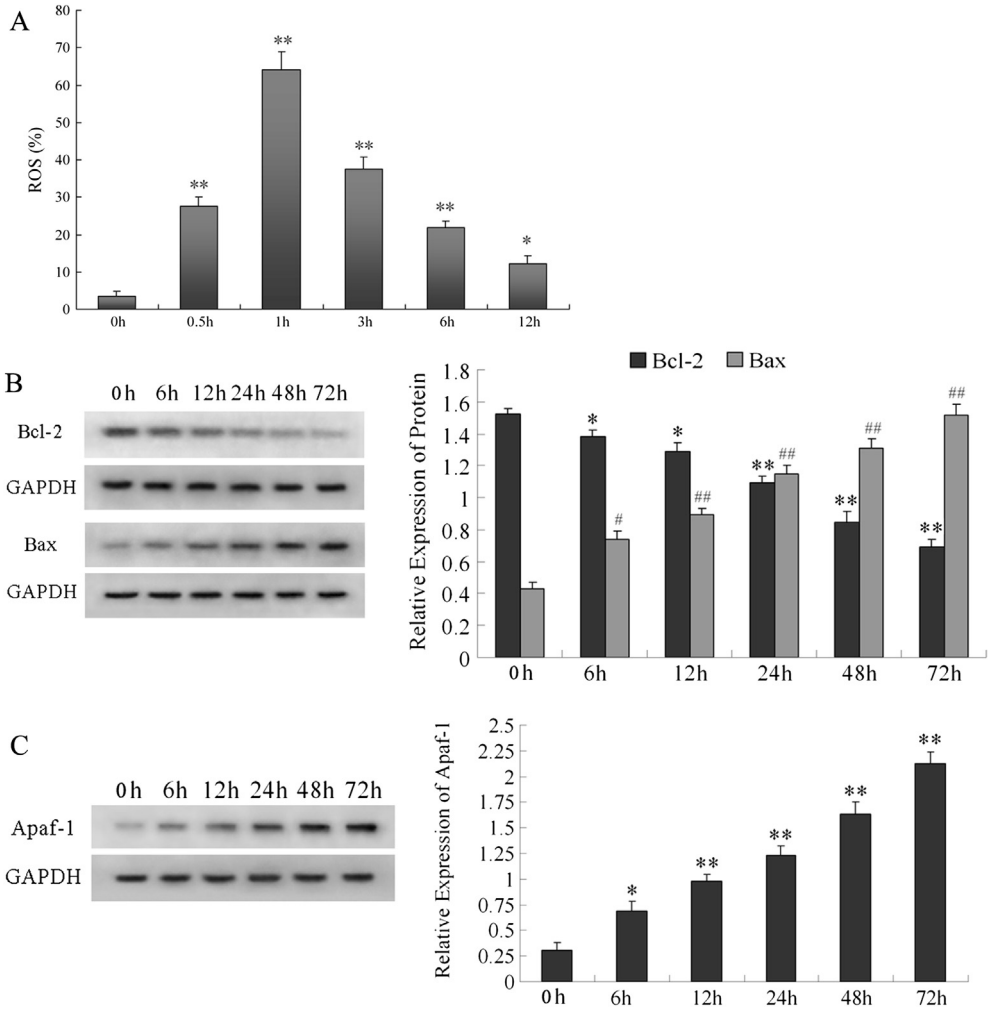

Fig. 3. (A) Flow cytometric analysis of ROS levels in rat C6 glioma cells with EMAP II treatment. (B) Effect of EMAP II treatment on protein expression of Bcl-2, Bax and Apaf-1 (C). Values represented the means ± SD (n = 6, each). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. EMAP II 0 h group of ROS, Bcl-2 and Apaf-1; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs EMAP II 0 h group of Bax.

morphological changes involving cell shrinkage, condensed or fragmented nuclei were observed. Those proved that EMAP II inhibits the viability of C6 glioma cells by inducing apoptosis. Two major apoptotic pathways have been well characterized. One pathway is the cell death receptor-mediated pathway, and the second apoptosis signal pathway is the mitochondrial-mediated apoptotic pathway, which is activated by various stimuli such as DNA damage and most types of chemotherapeutic agents [15]. Chemotherapy and radiotherapy used to kill tumor cells are principally mediated by the process of apoptosis that is governed by the mitochondria. The failure of anticancer therapy often resides at the level of the mitochondria [16]. Therefore, we detected whether the mitochondrial pathway was involved in EMAP II inducing apoptosis of C6 glioma cells. The mitochondrial integrity is disrupted as a result of changes in the MMP and release of cytochrome *c* from the mitochondria into the cytosol [17]. In the cytosol, cytochrome *c* binds to Apaf-1, in concert with the initiator caspase-9, forms the apoptosome. Activated caspase-9 directly cleaves and activates the executioner caspase-3, consequently leading to cell apoptosis [18]. Our results showed that EMAP II decreased the levels of MMP and promoted the cytochrome *c* released from mitochondria followed by activation of caspase-9 and caspase-3 in rat C6 glioma cell. This demonstrated that EMAP II could induce C6 glioma cell apoptosis through mitochondrial-mediated pathway.

Intracellular ROS functions as a trigger of signaling molecules to initiate downstream events in regulating cell cycle, cell differentiation and apoptosis [19]. However, excessive ROS production would impair and oxidize DNA, lipids, sugars and proteins and consequently result in dysfunction of these molecules within cells and finally cell death. Mitochondria not only lie in the center of the cell apoptotic pathway, but also are the major source of ROS generation [20]. These researches indicate that dysfunction of the mitochondria can induce cell apoptosis via producing ROS. Nevertheless, whether ROS is involved in the processing of EMAP II inducing rat C6 glioma cells apoptosis through mitochondrial pathway is unclear. Increasing evidence suggests that elevation in intracellular Ca^{2+} may lead to ROS generation [21,22]. It is reported that EMAP II increased the concentration of intracellular Ca^{2+} in endothelial cells [23]. Those researches suggest that EMAP II might up-regulate ROS level. Our result showed that EMAP II increased the level of ROS, and the most obvious changes appeared at 1 h. The time course was prior to the changes of MMP, cytochrome *c*, caspase-9 and caspase-3. This suggested that ROS played an important role during EMAP II induced rat C6 glioma cells apoptosis via mitochondrial pathway.

Bcl-2 family members are the important apoptosis regulatory factors and constitute critical control points in the mitochondrial pathway. The pro-apoptotic protein Bax and the antiapoptotic protein Bcl-2 are two of the most important members concerning apoptosis in Bcl-2 family [24]. Our data showed that EMAP II greatly inhibited the expression of Bcl-2 and promoted the expression of Bax in rat C6 glioma cells, that is to say, EMAP II could increase the ratio of Bax/Bcl-2. Studies demonstrate that Bcl-2 acts primarily to preserve MMP, suppresses the release of cytochrome *c* and apoptosis inducing factor [25]. Bax can permeabilize the outer mitochondrial membrane and then induce redistribution of cytochrome *c* from the mitochondrial intermembrane space into the cytoplasm, where it causes activation of caspase proteases (for instance caspase-9) and, subsequently, cell death [26]. Hence, we concluded, on the basis of the results mentioned above, that Bcl-2 and Bax might be involved in the modulation of EMAP II inducing rat C6 glioma cells apoptosis. Moreover, evidences revealed that ROS might result in the elevation in the ratio of Bax/Bcl-2 or the expression of Bax, and then induce apoptosis [27,28]. Our result showed that time course change of ROS was prior to the elevation of the Bax/Bcl-2 ratio. Therefore, a possible mechanism in EMAP II-induced apoptosis in rat C6 glioma cells is involved in ROS elevation, which is serving as a pivotal function, and then leads to an increase in the Bax/Bcl-2 ratio, ultimately leading to the activation of caspase-3.

In the present study, we demonstrated that EMAP II induces the apoptosis of rat C6 glioma cells in a time-dependent manner and the possible mechanism is associated with the mitochondrial pathway. Moreover, our previous studies showed that EMAP II could increase the BTB permeability through the paracellular and transcellular pathway. These results suggested that EMAP II might have dual effects on the therapy of brain tumor. On the one hand, EMAP II could open the BTB selectively, and contribute delivery of anti-tumor agents to brain tumors. On the other hand, EMAP II could induce apoptosis of rat C6 glioma cells directly. These findings will help to further elucidate the mechanisms of EMAP II inhibiting the tumor growth.

Declaration of financial disclosure

The authors have no financial conflict of interest.

Conflicts of interest

None.

Acknowledgments

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