Tamoxifen-induced apoptosis of rat C6 glioma cells via PI3K/Akt, JNK and ERK activation

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Abstract. To elucidate the mechanism of TAM treatment on gliomas, we hypothesised that PI3K/Akt and MAPK signaling pathway may play important roles on TAM-induced apoptosis in C6 glioma cells. Our results demonstrated that TAM induced apoptosis of C6 glioma cells in a dose-dependent manner. The activation of AKT significantly decreased in a time-dependent manner in response to TAM treatment, JNK was transiently activated, and subsequently decreased activation and kept stable level, whereas ERK evidenced sustained activations in response to the drug treatment. The inhibition of PI3K/Akt and JNK both accelerated and enhanced TAM-induced apoptosis and ERK inhibition apparently exerted negative effect on apoptosis. We also observed that PI3K/Akt had intimate association with JNK and ERK activation in TAM-induced apoptosis. These findings may provide strategies for the molecularly targeted therapy in malignant gliomas.

Introduction

Malignant gliomas are relatively uncommon but lethal cancers. The current standard treatments for malignant gliomas include surgical resection, radiation therapy and chemotherapy. When feasible, maximal surgical resection of tumors improves survival. Until recently, radiation has been the main standard-of-care treatment with a minimal role for systemic chemotherapy. However, adjunctive chemotherapy (concurrent chemotherapy medicine with radiation) improves median survival distinctively compared with radiation therapy alone (1).

Tamoxifen (TAM), a member of the selective estrogen receptor modulator (SERM) family, is widely used in the treatment of ER^+ breast cancers. But in recent years, high dose TAM (4-8 fold higher than those used for ER^+ breast cancers) has shown clinical benefit as a chemotherapy medicine in treatment of ER^- tumors, including glioma (2-4). Although a variety of previous studies have indicated that several survival-related or death-associated signaling pathways are involved in the effect of TAM on gliomas (5,6). However, the mechanisms were not fully understood, and only a very limited amount of information is available regarding the regulation and function of the PI3K/Akt and mitogen-activated protein kinases (MAPKs) pathways in TAM-induced apoptosis in gliomas.

The PI3K/Akt signaling pathway might be the best characterized and most illustrious pathway in the transmission of anti-apoptotic signals in cell survival. Akt is a key mediator of PI3K signaling located at an intersection of multiple pathways implicated in cell proliferation, survival, transcription, and metabolic processes (7,8). The MAPKs including the ERK1/2, JNK, and p38 MAPK subgroups, are also known to play fundamental roles in survival, proliferation, and apoptosis (9). We have known that the activation of ERK may play roles in the apoptosis induced by TAM in C6 glioma cells (10), but the functions of JNK, PI3K/Akt and the relationship between PI3K/Akt and MAPK still are not known. In this study, we have examined the regulation and function of PI3K/Akt and MAPK signaling pathways in tamoxifen-induced apoptosis in C6 glioma cells and the interplay between the two signaling pathways. Our results showed that the PI3K/Akt pathways and JNK exerted a negative regulatory effect in TAM-induced C6 glioma cell apoptosis, while the activation of ERK appeared to be positively related to apoptosis. Our findings indicated that PI3K/Akt mediated ERK and JNK in TAM-induced apoptosis in C6 glioma cells. Collectively, our results underlined the notion that interplay among the Akt signaling pathways, the activation of JNK and ERK plays a crucial role in C6 glioma cell apoptosis induced by TAM.

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Abbreviations: TAM, tamoxifen; ER, estrogen receptor; PI3K, phosphoinositide 3-kinase; JNK, c-Jun amino-terminal kinase; ERK1/2, extracellular signal-regulated kinase 1/2; MAPK, mitogen-activated protein kinase; FBS, fetal bovine serum; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulphate; PBS, phosphate-buffered solution; DMSO, dimethyl sulfoxide; FACS, fluorescence activated cell-sorting; PI, propidium iodide

Key words: glioma, apoptosis, tamoxifen, PI3K/Akt, signaling pathway

Materials and methods

Materials. TAM (Sigma-Aldrich) stock solution (10 mM) was prepared in analytical DMSO and stored at -20˚C until needed. The specific inhibitor of AKT LY294002 were acquired from Sigma-Aldrich (USA), JNK inhibitor SP600125 and MEK inhibitor PD98059 were obtained from Calbiochem (La Jolla, CA, USA). Rabbit polyclonal antibodies against phospho-AKT (Ser473) or AKT were purchased from Keygen (Nanjing, China). Antibodies against ERK1/2, phospho-ERK1/2 (Thr-202/Tyr-204), phospho-JNK were obtained from Cell Signaling Technology (Cummings Center, Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibody was obtained from Multisciences (Beijing, China). Enhanced chemiluminescence (ECL) kit was obtained from GE Healthcare (USA).

Cell culture and treatment. Rat C6 cell line was a gift from Professor Wenlin Huang (Sun Yat-sen University, China, Purchased from ATCC). Cells were maintained in Dulbecco's modified medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere of 5% CO_2 in 25-cm^2 culture flasks (Corning). When cells became 90% confluent, they were subcultured.

For the experiments, the cells were trypsinized with 0.125% trypsin-0.02% EDTA, stained by trypan blue exclusion for viability and seeded into 25-cm^2 culture flasks (Corning) at a density of 3-5x10^5 cells/ml in medium supplemented with 10% FBS. After overnight incubation, the medium was replaced with serum-free medium for additional 24 h and then changed to fresh serum-free medium with various concentrations TAM in different experiments. For inhibition experiments, quiescent cells were treated with 20 μM of LY294002, or SP600125 or PD98059 for 1 h and with additional 20 μM TAM for another 1 h followed by harvest of the cells for Western blotting.

Cell count. Cells were plated at a density of 4x10^5 cells in 24-well plates, then treated with TAM at 10, 20, 30, 40 μM for 24 h or 0.3% DMSO for vehicle control. Adherent cells were harvested by 0.125% trypsin-0.02% EDTA, combined with detached cells. Cell suspension (10 μl) was mixed with 90 μl Typan Blue dye exclusion medium in 1.5-ml centrifuge tube, then the hemacytometer chamber was carefully filled evenly and left undisturbed for 1-2 min. Then observed under an inverted phase contrast microscope to count live and dead cells, respectively, by hemacytometer, and the death rate was established.

DAPI staining. The cells were grown on coverslips in 6-well plates. After treatment the cells were fixed in 4% paraformaldehyde for 15 min at room temperature before permeabilization with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min. Cells were then rinsed with PBS and incubated for 15 min at room temperature with 0.01% DAPI stain (Sigma-Aldrich). Excess stain was removed by washing three times in PBS. Stained nuclei were visualized using a Leica fluorescent microscope. The cells with condensed and/or fragmented nuclei were scored as apoptotic cells.

Western blotting. The treated cells were washed twice with cold PBS and lysed in extraction buffer (20 mM Hepes, pH 7.4, 2 mM EDTA, 50 mM ß-glycerophosphate, 1 mM dithiothreitol, 1 mM Na_3VO_4, 1% Triton X-100, and 10% glycerol) on ice. The lysates were centrifuged at 12,000 rpm for 15 min. Supernatants were collected and protein concentrations were determined by BCA Protein Assay (Bio-Rad). Proteins were heated with sample buffer, separated in 12% SDS-polyacrylamide gel by electrophoresis (50 μg/lane) and electroblotted onto nitrocellulose membrane (Pall Corporation, Ann Arbor, MI, USA). Transferred blots were incubated sequentially with blocking agent (5% non-fat milk in TBS), rabbit anti-phospho-AKT, anti-phospho-JNK, anti-phospho-ERK1/2 antibodies and secondary antibody. Blots were developed by the enhanced chemiluminescence detection kit on Hyperfilm (Fuji, Japan) according to the manufacturer's directions. The same blots were subsequently stripped and reblotted with antibodies that recognized total Akt, total JNK or total ERK1/2 to verify equal amounts of the protein in various samples. Graphs of blots were obtained in the linear range of detection and were quantified for the level of specific induction by Scion Image System.

Quantification of apoptosis by fluorescence activated cell-sorting (FACS). C6 cells were treated with TAM at 10, 20, 30, 40 μM for 24 h or 0.3% DMSO as vehicle control. Adherent cells were harvested by 0.125% trypsin-0.02% EDTA, combined with detached cells, and fixed in 70% cold ethanol overnight. The cells were subsequently treated with propidium iodide (50 mg/ml) and RNase (20 mg/ml) for 15 min, and protected from light before analysis using a flow cytometer (Beckman) equipped with an air-cooled argon ion laser emitting at a wavelength of 488 nm at 15 mw. DNA histograms were interpreted using Coulter cytological program from a minimum of 10,000 events per sample. In addition, C6 cells were treated with 20 μM of LY294002, or SP600125 or PD98059 for 1 h and with additional TAM at various concentrations for 24 h, and then harvested for quantitative analysis of apoptosis in inhibitor experiments.

Results

TAM-induced apoptotic cell death of C6 glioma cells. To determine whether TAM affects the viability and can cause apoptosis of C6 glioma cells, dead cell numbers were counted by hemacytometer chamber and apoptotic cells were determined by a flow cytometer after different concentration (0, 10, 20 30 and 40 μM for 24 h) of TAM treatment. As expected, TAM resulted in significant cells death and cell apoptosis. Treatment with TAM at 20 μM caused about half of the cells to die after 24 h (Fig. 1A and B). Similar results occurred in apoptotic cell death (Fig. 1C). After cells were treated with 20 μM TAM for 24 h, DAPI staining revealed condensed nuclei (Fig. 1D). Thus, in the subsequent experiment, we elected to employ 20 μM of TAM to treat C6 glioma cells.

TAM decreases activity of PI3K/AKT in C6 glioma cells. Although above data (Fig. 1) have demonstrated that TAM could induce apoptotic cell death of C6 glioma cells, the

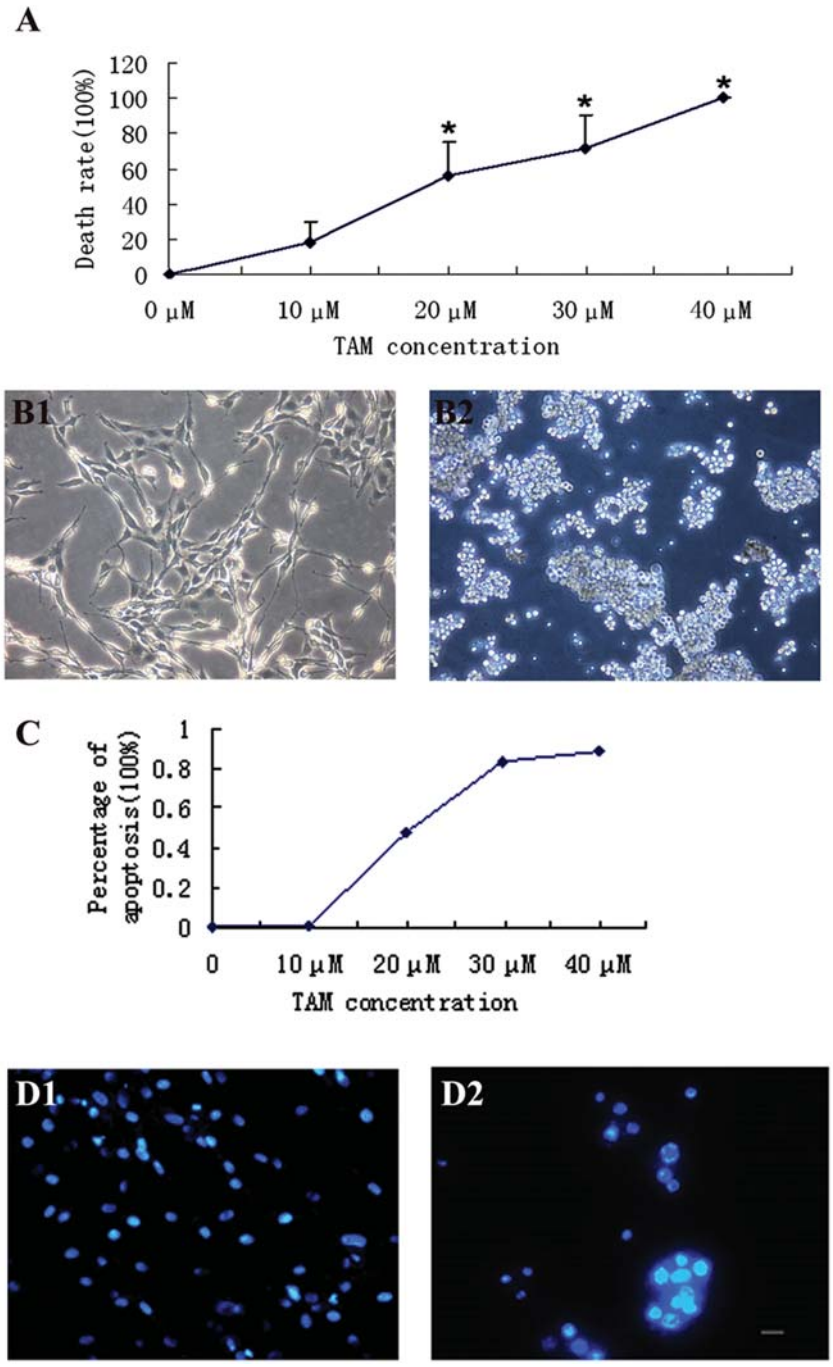

Figure 1. TAM induced apoptotic cell death of C6 glioma cells. C6 glioma cells were treated with different doses of TAM for 24 h as indicated. Cells treated with 0.3% DMSO were used as controls (0). (A) Cell death rate was determined via cell count. The data are expressed as the means ± SE of values from three independent experiments. (B) Normal C6 glioma cells (B1) and C6 glioma cells (B2) were treated with 20 μM TAM for 24 h (bar 100 μm). (C) The cells were harvested and stained with propidium iodide, and their percentage of apoptosis were analyzed via flow cytometry. (D) Normal C6 glioma cells (D1) and C6 glioma cells (D2) were treated with 20 μM TAM for 24 h, then stained with DAPI (bar 100 μm).

molecular mechanisms by which TAM induced C6 cell apoptosis are largely unknown. As PI3K/AKT was reported to take part in TRAIL-induced apoptosis in glioma cells (11), we then attempted to determine whether PI3K/AKT was involved in TAM-induced apoptosis in C6 glioma cells. It was noted that phosphorylation of AKT was significantly decreased in a time-dependent manner in response to TAM treatment (Fig. 2). After 6 h, the level of AKT phosphorylation declined gradually to basal levels. These results suggested that the down-regulated PI3K/AKT activation was involved in the TAM-induced apoptosis.

Differential regulation of JNK and ERK phosphorylation in C6 glioma cells. JNK is activated by several different anticancer drugs (12). However, its role in TAM-induced apoptosis in C6 cells remained somewhat unclear. Therefore, we have attempted to determine the effect of JNK phosphorylation in TAM-induced apoptosis in C6 glioma cells

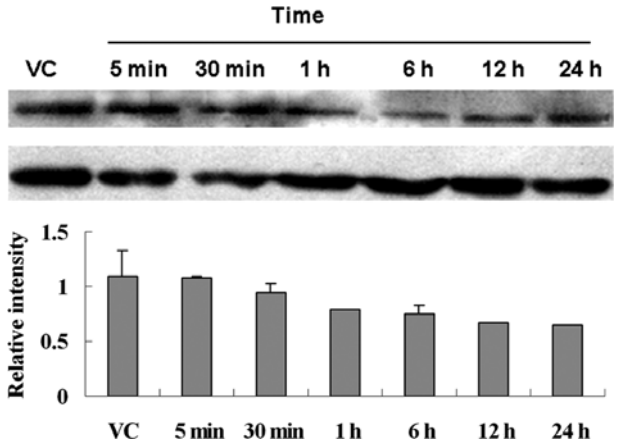

Figure 2. Time-dependent decrease of Akt phosphorylation in TAM-induced apoptosis. Cells were exposed to 20 μM TAM for indicated time periods. Cells treated with 0.3% DMSO were used as controls (VC). Proteins (50 μg) from total cell lysate were resolved via SDS-PAGE electrophoresis. The phosphorylation status of Akt was analyzed via Western blotting with anti-phospho-Akt. Equal protein loading was verified via the probing of the membranes with antibodies for the detection of total AKT.

short- and long-term. Our results showed that the level of phosphorylated JNK increased quickly and reached the peak at 1 h TAM treatment, and then slowly decreased, and stabilized at higher level compared with VC 6 h after TAM treatment (Fig. 3A).

The role of ERK in TAM-induced apoptosis in C6 glioma cells has been detected in our laboratory in short-term treatment (within 1 h) (10). Our past results showed that TAM treat-ment increased the phosphorylation of ERK1/2. In the current experiment, the phosphorylation of ERK1/2 was increased with the time of TAM treatment within 12 h and obviously declined, but was still much higher than VC after 24 h treatment (Fig. 3B). These findings strongly indicated that activated JNK and ERK1/2 may both be involved in the TAM-induced apoptosis of C6 glioma cells.

Effects of specific kinase inhibitors on TAM-induced apoptosis in C6 glioma cells. PI3K/Akt and MAPKs signaling pathways are clearly involved in a variety of cellular functions, including cell growth, differentiation, development, and apoptosis (13). In order to determine whether or not the PI3K/Akt, JNK and ERK signaling pathways are functionally involved in the apoptotic effects of TAM, we treated cells with several specific kinase inhibitors, including LY294002, SP600125, and PD98059, each of which blocks a specific PI3K/Akt or MAPK pathway. Treated with LY294002 (PI3K/Akt inhibitor) resulted in significant increases in TAM-induced apoptosis, and treated with SP600125 (JNK inhibitor) slightly enhanced apoptosis induced by TAM. At the same time, PD98059 (ERK 1/2 inhibitor) can inhibit TAM-induced apoptosis in C6 glioma cells. These results indicate that the activations of PI3K/Akt and JNK are functionally involved in the negative regulation of TAM-induced apoptosis in C6 glioma cells, whereas sustained ERK activation might be positively related to apoptosis (Fig. 4).

Interplay among PI3K/Akt, JNK and ERK in TAM-induced apoptosis. The above results showed that PI3K/Akt, JNK and ERK played roles in TAM-induced apoptosis. In order to confirm the interplay among PI3K/Akt, JNK and ERK in TAM-induced apoptosis, cells were treated with TAM and specific kinase inhibitors, respectively, or simultaneously, and then the phosphorylated PI3K/Akt, JNK and ERK1/2 were detected after 12 h. The results showed that when cells were treated with TAM and inhibitor at the same time, PI3K/AKT inhibitor LY294002 can increase the phosphorylation of ERK1/2 and JNK; JNK inhibitor SP600125 can decrease the phosphorylation of ERK, and ERK inhibitor PD 98059 also decrease the phosphorylation of JNK (Fig. 5).

Discussion

Several years of research on TAM at the cellular and molecular level have brought forward many signaling intermediates

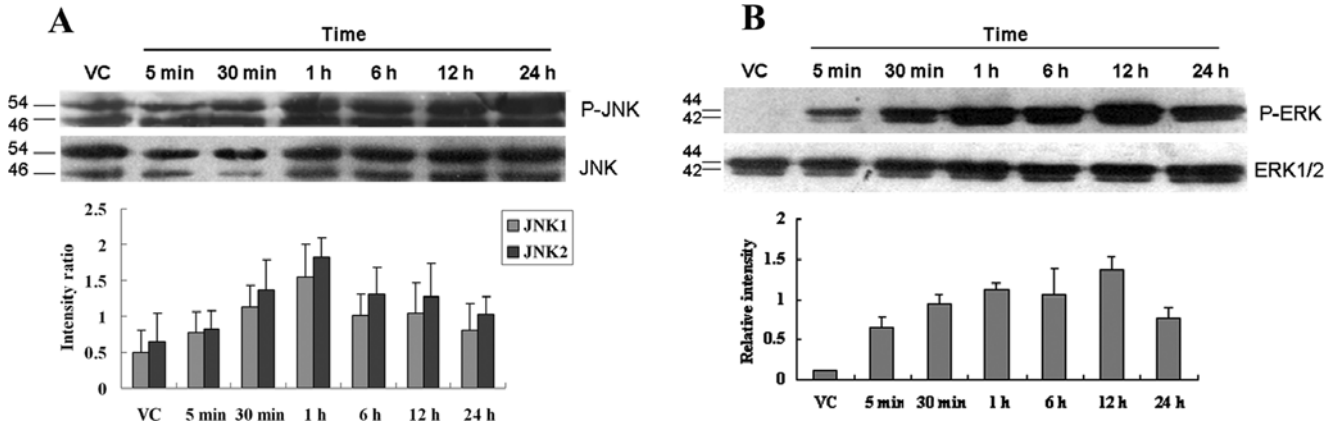

Figure 3. Time-course of phosphorylated JNK and ERK in TAM-induced apoptosis. Cells were exposed to 20 μM TAM for indicated time periods. Cells treated with 0.3% DMSO were used as controls (VC). Proteins (50 μg) from total cell lysate were resolved via SDS-PAGE electrophoresis. The phosphorylation status of JNK and ERK were analyzed via Western blotting with anti-phospho-JNK and anti-phospho-ERK1/2. Equal protein loading was verified via the probing of the membranes with antibodies for the detection of the respective anti-JNK and anti-ERK1/2.

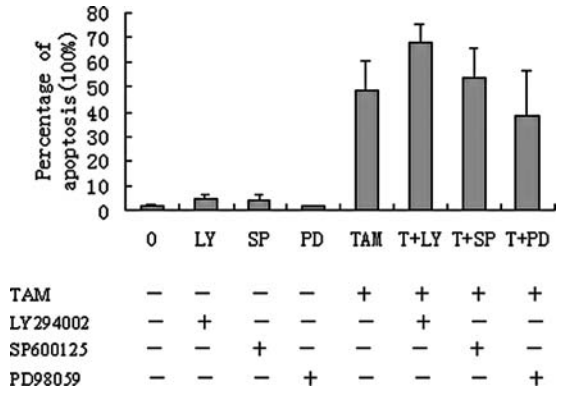

Figure 4. Effects of specific kinase inhibitors on TAM-induced apoptosis. C6 glioma cells were treated with a specific kinase inhibitor such as AKT inhibitor LY294002 (20 μM), JNK inhibitor SP600125 (20 μM) and ERK inhibitor PD98059 (20 μM) for 1 h and then treated for 24 h with additional TAM (20 μM). Cell death was analyzed via flow cytometry. The percentages of apoptotic cells are shown. Cells with 0.3% DMSO were employed as a control.

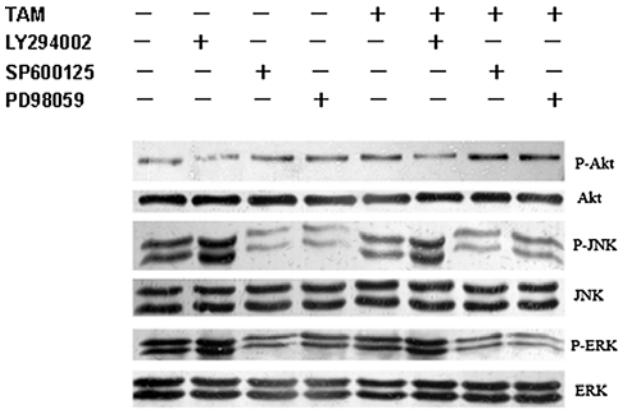

Figure 5. Interplay between PI3K/Akt, JNK and ERK on TAM-induced apoptosis. C6 glioma cells were treated with a specific kinase inhibitor such as AKT inhibitor LY294002 (20 μM), JNK inhibitor SP600125 (20 μM) and ERK inhibitor PD98059 (20 μM) for 1 h and then treated for 12 h with additional TAM (20 μM). Cells treated with 0.3% DMSO were used as controls (VC). The cells were lysed, and the cell lysates were resolved via SDS-PAGE. The phosphorylation status of Akt, JNK and ERK was analyzed via Western blotting with anti-phospho-Akt, anti-phospho-JNK and anti-phospho-ERK1/2. Equal protein loading was verified via the probing of the membranes with antibodies for the detection of the respective anti-Akt, anti-JNK and anti-ERK1/2.

that may play a role in its anticancer mechanism. TAM has been widely used in the treatment of estrogen receptor positive (ER) breast cancer for over a decade (14), but later it also was found to be efficient in treatment with ER-negative tumors such as malignant glioma and melanoma (15-17). Results from large amount of studies suggested induction of apoptosis might be a major mechanism of anti-tumor effect of TAM, which is achieved through a combination of ER-mediated and non-ER-mediated (or other signaling pathways) mechanisms. The signaling proteins in the non-ER-mediated pathways include protein kinase C(PKC), TGF-ß, c-myc, PI3K/Akt and MAP kinases *et al* (18).

In past studies, the signaling pathway including the calcium signaling (19), JNK1/caspase-3 (20), PKCε/ERK (21), JNK/c-Jun/AP-1/FasL (22) and PKCα/ERK1/2 (10) pathway have been found to take part in the anti-tumor effects of TAM on gliomas. But the roles of PI3K/Akt and MAPK in TAM-induced apoptosis in C6 glioma cells have not be studied as well as the correlations among PI3K/Akt, MAPK and TAM on glioma cells. In this study, we have conducted an investigation into the functions of PI3K/Akt, JNK and ERK 1/2 pathways in the context of the mechanism of TAM-induced apoptosis on C6 glioma cells and relationships among them. Our data may offer therapeutic benefit in the TAM treatment of various cancers including both ER^+ and ER^- malignancies.

The PI3k/Akt cellular pathway is particularly relevant to proliferation and cell survival in malignant glioma in which its constitutive activation results from the loss of function of PTEN/MMAC1. Activation of this pathway by phosphorylation and functional inactivation of several pro-apoptotic targets, including the BCL-2 family member BAD and the protease caspase-9 *in vitro* protected glioma and other malignant cells against apoptosis (23,24). Many malignant glioma cell lines, including C6 glioma cells, constitutively expressed activated Akt (25,26). Our results were consistent with the investigations on the roles of PI3K/Akt on glioma (27). In the context of TAM, the expression of phosphorylated Akt decreased in a time-dependent manner in C6 glioma cells, and inhibitor of PI3K/Akt signaling (LY294002) promoted the apoptosis induced by TAM significantly, indicating the anti-apoptotic roles of Akt activation in TAM-induced apoptosis on C6 glioma cells.

MAPK are proline containing serine-threonine kinases that are organized in a cascade of three sequential phosphorylations, resulting in activation (28). The three members of the MAPK family, JNK, p38 and ERK, have been implicated in distinct cellular processes. In general, JNK and p38 MAPK are activated by diverse stimuli such as oxidative stress, UV irradiation, and osmotic shock and required for the induction of apoptosis (29). In contrast, ERK plays vital roles in cell growth and division and is generally considered to be a survival mediator. However, the detailed functions of these MAPK members depend on the type of stimuli and cell systems (30). Studies have also shown that the MAPK signaling pathway plays an important role in the action of chemotherapeutic drugs (31).

JNK was originally identified as two protein kinases, p46 JNK1 and p54 JNK2 both of which specifically phosphorylate the transcription factor, c-Jun, at the Ser 63 and Ser 73 of its N-terminal transactivation domain (32,33). JNK is activated by a host of cytotoxic drugs, and this activation generally contributes to the mediation of drug-induced cell death (34). The significance of such activation in the induction of apoptosis is uncertain, but it appears to depend on the particular pathway that is involved, the duration of activation, as well as the cell type (5). Tseng *et al* investigated the role of JNK1 and caspase-3 in TAM-induced apoptosis of rat glioma cells. Their results showed increased phosphorylation of JNK1 and all of the TAM-induced effects were blocked by inhibition of JNK1 (20). Those data suggested that JNK1 might play a key role in facilitating

tamoxifen-induced apoptosis in these tumor cells. However, it could be due to overlapping or redundant functions of JNK1 and JNK2 (35). Our results showed TAM activated JNK in a biphasic manner with an early transient increase (within 1 h) and then decrease, followed by stabilization at a higher phosphorylation level comparing to control cells. The inhibitor of JNK (SP600125) slightly promote the apoptosis induced by TAM. Our data indicated that the activation of JNK was a relatively early transient event in the response of C6 cells to TAM, and its ultimate effect was slightly anti-apoptotic, which corresponded with the studies (36,37) that inhibition of JNK activity has been shown to cause growth arrest or apoptosis in certain types of cells.

Although the activation of the ERK1/2 pathway is normally associated with cell proliferation and survival, a number of studies showed that depending on the stimuli and cell types involved, activation of ERK1/2 can mediate cell death (38-40). Some studies suggest that the balance among the intensity and duration of pro- vs. anti-apoptotic signals transmitted by ERK1/2 determines whether a cell proliferates or undergoes apoptosis (41). Our study consistently demonstrated that TAM-induced apoptosis almost sustained ERK1/2 activation, and the suppression of ERK by PD98059 treatment appeared to exert a significant inhibition on TAM-induced apoptosis.

MAPK and PI3K/Akt are two of the most important signaling pathway frequently dysregulated in cancer. In addition, there is accumulating evidence that two pathways may cooperate to promote the survival of transformed cells (33,42-44), but the interactions among them remain to be resolved. Some studies indicated that ERK1/2 located downstream of Akt and overexpression of Akt apparently suppressed the phosphorylation of ERK1/2 (33,45,46). Also there were some experiments showing inhibition of Akt can increase the phosphorylation of JNK. In our study, inhibition of Akt activation resulted in an increase in ERK1/2 and JNK phosphorylation. This suggested that the activation of the ERK1/2 and JNK signaling pathway might be regulated negatively by the PI3K/Akt signaling pathway in TAM-induced apoptosis, which is consistent with other studies on the relationship among ERK1/2, JNK and PI3K/Akt.

In conclusion, our study suggested that PI3K/Akt, JNK and ERK 1/2 all play important roles in TAM-induced apoptosis in C6 glioma cells. The activation of PI3K/Akt and JNK promotes apoptosis, and ERK 1/2 activation might be associated negatively with the apoptosis induced by TAM. At the same time, there was interplay among PI3K/Akt, JNK and ERK 1/2 during apoptosis. JNK and ERK1/2 might be regulated negatively by the PI3K/Akt signaling pathway in C6 glioma cells. Our findings might provide better insight into the mechanism of TAM-induced apoptosis in gloma cells, which could contribute to the development of molecularly targeted therapy for malignant gliomas.

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