Proliferation arrest and induction of CDK inhibitors p21 and p27 by depleting the calcium store in cultured C6 glioma cells

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In this study, we investigated the role of the intracellular calcium store in modulating the cellular proliferation and the expression of cell cycle regulatory proteins in cultured C6 glioma cells. By means of microspectrofluorimetry and Ca^{2+}-sensitive indicator fura-2, we found that the intracellular Ca^{2+} pump inhibitors, thapsigargin (TG) irreversibly and 2,5-di-tert-butyl-hydroquinone (DBHQ) reversibly depleted the Ca^{2+}-store accompanied with the induction of G_0/G_1 arrest, an increase in glial fibrillary acidic protein (GFAP) expression and morphological changes from a round flat shape to a differentiated spindle-shaped cell. The machinery underlying these changes induced by Ca^{2+}-store depletion was investigated. The results indicated that Ca^{2+}-store depletion caused an increased expression of p21 and p27 proteins (cyclin-dependent kinase inhibitors), with unchanged mutant p53 protein of C6 cells but reduced amounts of the cell cycle regulators: cyclin-dependent kinase 2 (CDK2), cdc2, cyclin C, cyclin D1, cyclin D3 and proliferating cell nuclear antigen (PCNA) in a time-dependent manner. These findings indicate a new function of the endoplasmic reticulum (ER) Ca^{2+} store in regulating cellular proliferation rate through altering the expression of p21 and p27 proteins. Moreover, cellular differentiation as revealed by spindle-shaped morphology and induced GFAP expression were also modulated by the ER Ca^{2+} store. The implication of this finding is that the abnormal growth of cancer cells such as C6 glioma cells may be derived from a signalling of the ER which can be manipulated by depleting the Ca^{2+} store.

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Abbreviations. CDK Cyclin-dependent kinase. – DBHQ 2,5-Di-tert-butylhydroquinone. – ER Endoplasmic reticulum. – GFAP Glial fibrillary acidic protein. – $InsP_3$ Inositol 1,4,5-trisphosphate. – MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. – PCNA Proliferating cell nuclear antigen. – TG Thapsigargin. – TGF Transforming growth factor. – TPA Phorbol 12-myristate 13-acetate.

Introduction

Cancer cells that grow uncontrollably, such as brain glioma, are largely resistant to chemotherapy [38, 48]. One of the best strategies for cancer management is to inhibit cell proliferation and to convert the cancer cells to differentiated normal cells. The second messenger inositol 1,4,5-trisphosphate ($InsP_3$) generates cytosolic Ca^{2+} signals by activating Ca^{2+} release from Ca^{2+}-sequestering target organelles, mediating a multitude of cellular responses [3, 37]. In response to signals mediated by growth factors and hormones, these pools of Ca^{2+} are transiently depleted by activation of the $InsP_3$-dependent Ca^{2+} channel in the endoplasmic reticulum (ER) membrane [9]; upon termination of the $InsP_3$-generating signal, the pools are able to refill via Ca^{2+} pumping activity [10]. In contrast to transient agonist-mediated Ca^{2+} pool depletion, thapsigargin (TG), a naturally occurring sesquiterpene lactone and non-phorbol ester-type tumor promotor, is a selective inhibitor of the intracellular Ca^{2+} pump [44, 46] and thus is capable of inducing a highly persistent depletion of $InsP_3$-sensitive Ca^{2+} pools [4, 18, 30].

Previous studies with mammalian and yeast systems demonstrated that a family of cyclin-dependent kinases (CDKs) are key regulators of cell progression [7, 35, 41]. The activities of these protein kinases are dependent on their association with different subsets of cyclin subunits whose expression oscillates

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during the cell cycle. The activity of CDKs is regulated by various mechanisms including association with cyclins, phosphorylation/dephosphorylation and association with a group of inhibitory proteins collectively called CKIs (cyclin-dependent kinase inhibitors) [32, 42]. The discovery of cyclin-CDK inhibitory proteins has provided a new paradigm for the control of cell growth which links the biochemical events surrounding cell cycle arrest with various physiological processes including the cellular response to DNA damage, differentiation, contact inhibition, senescence, response to growth inhibitory signals and suppression of tumorigenesis [12, 42]. The first of these negative regulatory proteins identified was the p53-activated DNA damage-inducible p21WAF1/CIP1 protein, a universal inhibitor of cyclin-CDK complexes and DNA replication [16, 51]. In addition to direct transcriptional induction by p53, various other signals have been reported to induce p21 expression in the absence of wild-type p53 [29]. Another CDK inhibitor p27 also has a broad specificity for CDKs. Expression of p27 by transient transfection arrests cells in G_1 [36, 47]. In p27-arrested Rat1 cells, p27 was stably associated with cyclin E/CDK2 complexes and inhibited their catalytic activity [50].

In the present study, using TG and a distinct fully reversible Ca^{2+} pump inhibitor, 2,5-di-tert-hydroquinone (DBHQ) [31], we demonstrated that these two compounds not only resulted in a profound inhibition of cell proliferation but also induced changes of cellular morphology from round shape toward a differentiated spindle in cultured C6 glioma cells. Furthermore, we found that a depletion of the intracellular Ca^{2+} store provoking a signal to increase the production of p21 and p27 proteins was the most probable mechanism for this action. We suggest that the signaling pathway of intracellular Ca^{2+} store depletion/CKIs production plays a role in regulating cell growth and differentiation. Thus, abnormal growth of C6 glioma can be effectively managed by a calcium store-depleting agent through this growth regulatory pathway.

Materials and methods

Chemicals

Thapsigargin was purchased from RBI (Natick, MA). Antibiotics, Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were from GIBCO (Gaithersburg, MD). Culture dishes and plates were from Costar (Cambridge, MA). Fura-2/AM and DBHQ were purchased from Sigma (St. Louis, MO). Rabbit polyclonal antibody to glial fibrillary acidic protein (GFAP) and FITC-conjugated goat anti-rabbit antibody were from ZYMED (South San Francisco). Goat polyclonal antibody to p21 (sc-397-G), rabbit polyclonal antibody to cyclin C (sc-1061) and mouse monoclonal antibody to cyclin D1 (sc-246) were from SANTA CRUZ (California). Mouse monoclonal antibody to p53 (OP03) was from Oncogene (Cambridge, MA). Mouse monoclonal antibody to p27, cdc2, CDK2, cyclin D3 and PCNA were from Transduction Laboratory (Lexington, KY). Other chemical drugs were purchased from Sigma (St. Louis, MO).

Cell lines and culture conditions

Glial C6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal calf serum, penicillin (50 IU/ml), streptomycin (50 μg/ml) under humidified atmosphere of 5 % CO_2 at 37 ± 0.5 °C. The cells were passaged when confluent by using trypsin-EDTA (0.05 %). Cells for experiments were seaded on coverslips or dishes and cultured for 1 day under the same conditions.

Measurements of intracellular calcium

Cells on coverslips were washed twice with the following mixture (buffer A): 154 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM Hepes (pH 7.2), 2.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM glucose. The cells were then incubated at room temperature with 2 μM fura-2/AM in buffer A for 40–50 min. Thereafter, the cells were washed three times with buffer A. Coverslips were mounted in a chamber over a Zeiss inverted-stage microscope equipped with a 40× oil-immersion fluorescence objective lens. All substances were added as solutions in buffer A. The cells were excited alternatively at 340 nm and 380 nm, whereas the emission intensity was measured at 520 nm. Digital fluorescent microscopy was used to determine the spatial distribution of $[Ca^{2+}]_i$. Experiments were evaluated on Photan Analysis System.

GFAP staining

Cells were grown on coverslips and washed three times with PBS and fixed in 100 % acetone at −20 °C for 5 min [25]. The cells were again rinsed three times with PBS and then incubated for 30 min with a primary polyclonal rabbit antibody raised against human glial fibrillary acidic protein (GFAP) at a 1:50 dilution. The cells were again rinsed in PBS followed by exposure to a second, FITC-conjugated goat anti-rabbit antibody at a 1:25 dilution for 30 min. After rinsing again in PBS followed by distilled water, coverslips were mounted using 1:1 glycerol:water and sealed with nail varnish.

DNA staining (cell cycle assay)

Cells grown on plates and treated with drugs for different times were harvested, washed, fixed in 70 % alcohol and stained with 5 μg/ml propidium iodide [17]. Fluorescence intensities were determined by FACSCAN flow cytometer (Becton Dickinson) and distribution of the cell cycle was analyzed.

Western blot analysis

From C6 cells treated with TG or DBHQ, cell lysates were prepared according to Jeoung et al. [19]. Each sample containing 60 μg of protein was applied to 15 % SDS-PAGE. Proteins transblotted to a nitrocellulose membrane were incubated with blocking buffer (1× PBS, 1 % BSA (w/v), and 0.2 % Tween 20 (v/v)) for 40 min at room temperature and washed with 1× PBST (1× PBS and 0.2 % Tween 20). The nitrocellulose membrane was incubated with primary antibody against p21, p27, p53, cdc2, CDK2, cyclin C, cycli D1, cyclin D3, or PCNA for one hour at room temperature and washed with 1× PBST for 20 min. The NC membrane was incubated with secondary antibody for one hour at room temperature, and after washing with 1× PBST for 20 min, detection of proteins of interest was carried out by the ECL method.

Results

Inhibition of cell proliferation and modification of cell cycle distribution

Thapsigargin (TG) and DBHQ exert a profound inhibitory effect on cellular growth and division. In spite of complete inhibition of cell growth, the cells remained intact and viable (trypan blue exclusion, MTT). As shown in Fig. 1, cell division is completely abolished when cells are treated with either 1 μM TG or 25 μM DBHQ for various time intervals from 1 to 4 days. As summarized in Table I, during a 4-day treatment with either 1 μM TG or 25 μM DBHQ, the number of cells remained essentially unchanged as compared to control cells, which increased 11- to 12-fold. Importantly, if the DBHQ was washed out of DBHQ-treated cells after 1-day culture, the cells rapidly resumed a normal proliferation rate and grew to >7-fold the original number during the 3-day period following washing. In contrast, after a similar 1-day treatment with TG followed by washing, there was no restoration of cell prolifera-

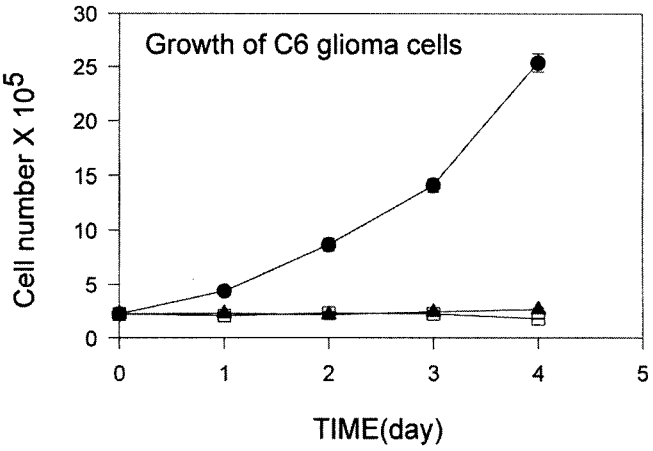

Fig. 1. Proliferation arrest induced by thapsigargin or DBHQ in cultured C6 glioma cells. Cells were cultured in growth medium for 12 h and then treated with either 1.0 μM TG (thapsigargin) (□) or 25 μM DBHQ (di-tert-butylhydroquinone) (▲) or without treatment (●) for various days as indicated. Cell viability of either control or drug-treated groups was > 95 %. Data (mean ± S.E.) shown are the result of a representative experiment, and more than five experiments showed similar results.

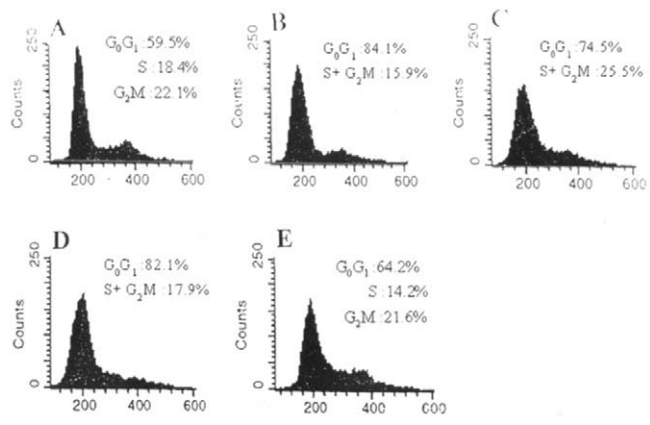

Fig. 2. Influence of TG and DBHQ on the cell cycle distribution of C6 glioma cells. Cells were grown for 1 day in growth medium. DNA frequency histograms (after staining with propidium iodide) are shown for cells from the control group (**A**) or from the groups treated with either 1 μM TG (**B**) or 25 μM DBHQ (**C**) for 3 days. Reversibility of the drug effects was tested by treatment with either 1 μM TG (**D**) or 25 μM DBHQ (**E**) for 2 days and then washing three times with medium followed by another 24 h culture. Results shown are from a representative experiment and were reproduced in four other identical experiments.

tion. The DNA content of C6 nuclei was measured by flow cytometry 24 h after the addition of TG and DBHQ. Cells treated with TG or DBHQ largely arrest in the G_0G_1 phase; the proportions of arrest at the G_0G_1 phase in the cells treated with either 1 μM TG or 25 μM DBHQ were 84.1 % and 74.5 %, respectively, as compared with 59.5 % for the control cells (Figs. 2A, B and C). In cells that had been treated with 1 μM TG for 2 days, followed by the washing out of TG and a subsequent one-day culture, the changes in the cell cycle distribution remained similar to those of unwashed TG-treated groups (Fig. 2D). In contrast, in cells treated with 25 μM DBHQ for 2 days, then washed with DBHQ-free PBS and cultured for another 24 h, the cell cycle distribution was shifted toward a pattern similar to that of the controls (Fig. 2E). We summarize the effects of TG and DBHQ on cell cycle distribution in Table II.

Tab. I. Reversibility of proliferation arrest induced by TG and DBHQ in cultured C6 glioma cells.

Treatment	Cell number ($\times 10^5$)[d]	Fold change in cell number
Control I[a]	2.23 ± 0.09	1.00
Control II[a]	25.37 ± 0.85[e]	11.38[e]
TG[b]	1.79 ± 0.04	0.81
TG; 24-h wash[c]	1.91 ± 0.07	0.86
DBHQ[b]	2.65 ± 0.15	1.19
DBHQ; 24-h wash[c]	17.65 ± 0.29[e]	7.91[e]

[a] Cells were passaged in growth medium in 24-well plates for 12 h without drug (Control I) and continued to culture for 4 days (Control II).
[b] Cells after 12-h culture were treated with either 1.0 μM TG (thapsigargin) or 25 μM DBHQ (di-tert-butylhydroquinone) for 4 days. Cell viability was > 95 %.
[c] TG- or DBHQ-treated cells were washed after 24 h of treatment and then continuously cultured in the inhibitor-free medium for another 3 days. Cell viability was > 95 %.
[d] (mean ± S.E)
[e] $P < 0.05$ as compared with that of Control I (ANOVA test).

Modification of cytosolic Ca^{2+} levels

In order to confirm that TG and DBHQ could initially increase intracellular free calcium and eventually depleted the intracellular calcium pool, we used a monolayer of C6 cells on coverslips loaded with fura-2/AM, and the effects of TG and DBHQ on cytosolic free Ca^{2+} levels were examined (Fig. 3A, B). TG and DBHQ induced a transient rise in $[Ca^{2+}]_i$, followed by an approximate 15–20 min return to the basal level. An interesting result was derived from conducting the same experiment but using cells that had been previously treated in culture with TG for 24 h (Fig. 3C). When TG was subsequently

Tab. II. Influence of TG and DBHQ on the cell cycle distribution of cultured C6 glioma cells.

Treatment	Cell cycle (%)	
	G_0G_1[d]	$S+G_2M$[d]
Control[a]	58.5 ± 0.42	41.8 ± 0.32
TG (no wash)[b]	85.1 ± 0.52[e]	15.2 ± 0.32[e]
TG (wash)[c]	83.9 ± 0.42[e]	17.2 ± 0.34[e]
DBHQ (no wash)[b]	79.2 ± 0.46[e]	21.2 ± 0.33[e]
DBHQ (wash)[c]	65.1 ± 0.39	36.9 ± 0.38

[a] Cells were grown for 1 day in growth medium.
[b] DNA frequency histograms of cells were analyzed after treatment with either 1 μM TG or 25 μM DBHQ for 3 days.
[c] Cells were treated with drugs for 2 days and then washed out of drugs, followed by a subsequent 1 day culture.
[d] (mean ± S.E.)
[e] $P < 0.05$ as compared with Control (ANOVA test).

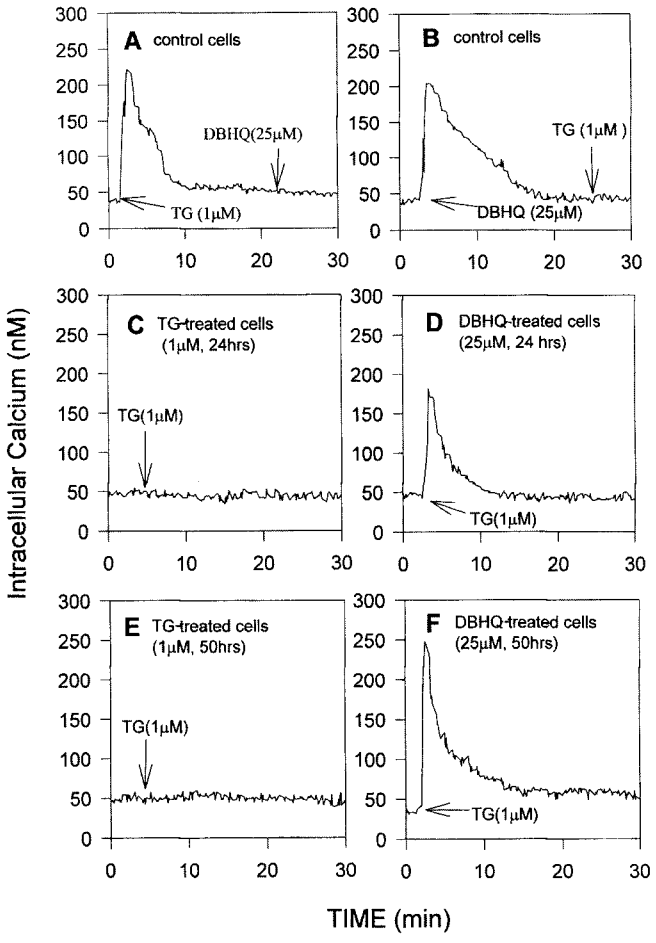

Fig. 3. Influence of TG and DBHQ on cytosolic free Ca^{2+} levels in C6 glioma cells. Cells were grown for 1 day in growth medium. Acute treatments with either 1 μM TG (**A**) or 25 μM DBHQ (**B**) were made to cells in the chamber at the time point indicated by the *arrow*. Prolonged treatment with either 1 μM TG (**C, E**) or 25 μM DBHQ (**D, F**) for either 24 h (**C, D**), or for 50 h (**E, F**) and subsequent washout of the drugs was performed prior to measurements of cytosolic free Ca^{2+}.

added to the cells after loading with fura-2/AM, there was no longer change in cytosolic Ca^{2+} level. By contrast, there was a transient increase in cytosolic Ca^{2+} level when TG was added to the cells that had been previously treated with 25 μM DBHQ for 24 h followed by washout and being loaded with fura-2/AM (Fig. 3D). After a longer treatment of 50 h leading to the changes of cellular morphology, the effect of TG on $[Ca^{2+}]_i$ was still irreversible (Fig. 3E) and that of DBHQ remained reversible (Fig. 3F), indicating that the Ca^{2+} pool was rapidly refilled after removal of DBHQ.

Clearly, both TG and DBHQ can readily enter intact cells and deplete intracellular Ca^{2+} pools. Following pool depletion by either agent, a second addition of a Ca^{2+} pumping inhibitor no longer induced a further release, indicating again that both inhibitors empty a single overlapping pool (Figs. 3A and B).

To obtain additional evidence to support the hypothesis that cellular proliferation arrest caused by TG and DBHQ is due to the Ca^{2+} store depletion rather than the initial increase in intracellular Ca^{2+} level, we have performed an experiment which showed that neither 1 μM A23187 nor 1 μM ionomycin could cause proliferation arrest (98±2% and 96±2%, respectively, n=4, $p>0.05$), although they certainly increase the Ca^{2+} level.

Induction of p21 protein and p27 protein in p53-independent manner

We performed Western immunoblot analysis to determine whether these Ca^{2+} pool depletion agents affect expression levels of p21 and p27 proteins in C6 cells. A short term treatment of C6 cells with 1 μM TG in Ca^{2+}-free medium (without FCS) for 20 min was carried out followed by washout and incubation in fresh medium for respective 6, 12 and 24 h, and finally cell lysates were loaded on SDS-PAGE. As shown in Fig. 4A, TG (lanes 2–4) was able to induce p21 protein as early as within 6 h and also to induce the expression of p27 protein after 12 h with a marked increase after 24 h, indicating that TG-increased p21 expression is not due to the influx of extracellular Ca^{2+} but is due to Ca^{2+}-store depletion. Another observation supporting this contention is that we cultured C6 cells in Ca^{2+}-free medium for 24 h which also depleted the Ca^{2+} store associated with cell proliferation arrest (55±4% of control, n=4, $p<0.05$). On the other hand, when C6 cells were treated with 1 μM TG or 25 μM DBHQ for 24 h and 48 h respectively in Ca^{2+}-containing medium and then cell lysates were electrophoresed, as shown in Fig. 4B, both TG (lanes 2 and 3) and DBHQ (lanes 5 and 6) were still able to induce p21 and p27 proteins. However, when C6 cells were treated with 25 μM DBHQ for one day, washed out and maintained for a subsequent 24 h, the cells returned to proliferation and expression of p21 and p27 protein was markedly reduced (Fig. 4B, lane 7) while those induced by TG persistently expressed both proteins even after washout (Fig. 4A, lane 4). The p53 protein, which was a mutant form [52] in C6 glioma cells, remained unchanged at a high level after treatment with either TG or DBHQ (Fig. 4B).

Modification of the cell cycle regulatory proteins

When C6 cells were induced by TG or DBHQ to arrest growth at G_0G_1 phase accompanied by the expression of CDK inhibitors (p21 and p27 protein), we examined whether the expression of other cell cycle regulatory proteins was also influenced. As shown in Fig. 5 (lanes 2, 3, 5 and 6), the expression of cdc2, CDK2, cyclin C, cyclin D1, cyclin D3 and PCNA was reduced in a time-dependent manner. When cells were treated with DBHQ for one day, washed out and maintained for another 24 h, the effects of DBHQ were abolished as revealed by the fact that these cell cycle regulatory proteins resumed to the normal levels (Fig. 5, lane 7), but the effects of TG were still irreversible after washout (Fig. 5, lane 4).

Morphological changes and enhanced expression of GFAP

In addition to the induction of proliferation arrest at G_0G_1 phase and the expression of CDK inhibitor proteins, TG and DBHQ also markedly caused the changes of cell morphology from a round flat shape (Fig. 6A) to an elongated differentiated spindle shape with bipolar processes (Figs. 6C, E). The cell morphology was markedly changed similar to that of differentiated cells induced by isoproterenol plus IBMX (data not shown). When cell division was arrested for one day and then the DBHQ was washed out, the morphology of the cells returned to the original round flat shape (Fig. 6F). In contrast, cells treated with TG followed by washing did not resume the original undifferentiated round flat shape (Fig. 6D).

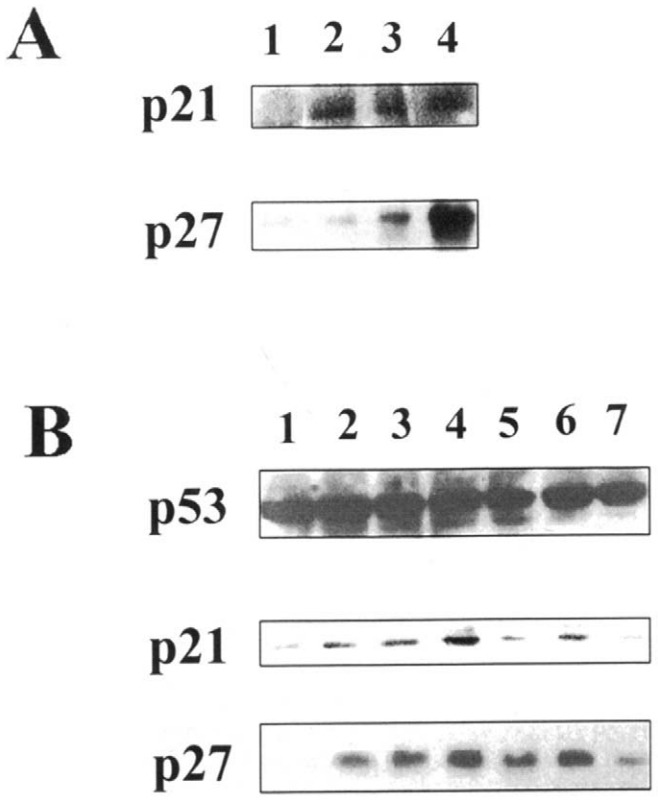

Fig. 4. Immunodetection of p21, p27 and p53 protein expression following TG and DBHQ treatment of C6 glioma cells. (**A**) C6 cells treated with or without (*lane 1*) 1 μM TG in Ca^{2+}-free medium for 20 min and then maintained in drug-free medium for 6 (*lane 2*), 12 (*lane 3*) and 24 h (*lane 4*). Cell lysates were then immunoblotted for p21 or p27 protein. (**B**) Control cells (*lane 1*) and cells treated with 1 μM TG (*lanes 2* and *3*) or 25 μM DBHQ (*lanes 5* and *6*) for 24 or 48 h, respectively. Reversibility of drug effects was tested by treatment with TG (*lane 4*) or DBHQ (*lane 7*) for one day and then washout by another 24 h culture in drug-free medium. Cell lysates were then immunoblotted for p53, p21 and p27 proteins.

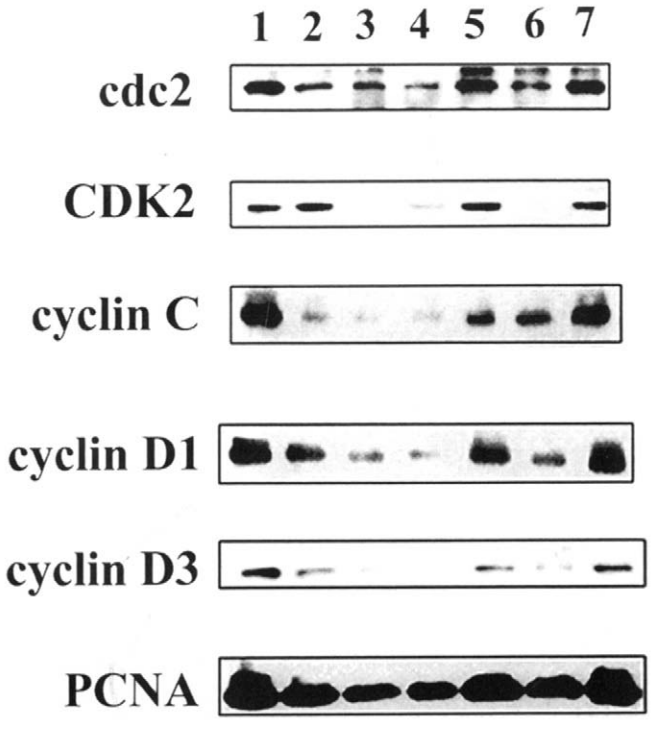

Fig. 5. Western blot analysis of cdc2, CDK2, cyclin C, cyclin D1, cyclin D3 and PCNA protein expression following TG and DBHQ treatment of C6 glioma cells. Shown are control cells (*lane 1*) and cells treated with 1 μM TG (*lanes 2* and *3*) or 25 μM DBHQ (*lanes 5* and *6*) for 24 or 48 h, respectively. Reversibility of drug effects was tested by treatment with TG (*lane 4*) or DBHQ (*lane 7*) for one day and then washout by another 24 h culture in drug-free medium. Cell lysates were then immunoblotted for cdc2, CDK2, cyclin C, cyclin D1, cyclin D3 and PCNA proteins, respectively.

We also utilized GFAP staining to reveal the change of cell morphology and cytosolic distribution of GFAP. When the control C6 cells were stained with GFAP antisera, most of the cells showed faint fluorescent staining (Fig. 7A). After 2 days of TG treatment, the majority of the cells were GFAP-positive (Fig. 7B); brightly stained long processes were observed in most of the cells. Perinuclear staining and diffuse staining of the cytoplasm were also observed in many cells, with a subtle, complex network of GFAP-positive intracytoplasmic filaments. Treatment with DBHQ for 2 days also enhanced the expression of GFAP protein similar to TG with an exception of reversible effects after washout (data not shown). GFAP staining showed that treatment with TG and DBHQ induced C6 glioma cells toward differentiation state.

Discussion

In this paper, we have demonstrated that the ER Ca^{2+} pump inhibitors, TG and DBHQ are potent inhibitors of cell proliferation in cultured C6 glioma cells. More than 80% of the cells entered G_0/G_1 phase and the cell morphology changed from a round flat shape to the differentiated type: long spindle with many protrudings similar to those induced by the differentiating agent, isoproterenol plus IBMX. The differentiation marker GFAP is markedly increased all over the cell body and the cell processes. The machinery underlying these profound effects was elucidated in this study.

Since TG and DBHQ are irreversible and reversible ER-Ca^{2+} pump inhibitors, respectively, we examined their reversibility on the depletion of intracellular the Ca^{2+} store in association with their effects on proliferation arrest. The results obtained confirmed our working hypothesis that Ca^{2+} plays a pivotal role in determining cell growth and differentiation; a good correlation was obtained between intracellular calcium depletion state and induction of the proliferation arrest. This finding is in agreement with the previous findings that Ca^{2+} exerts an important control over cell cycle events [49] perhaps mediated by the signaling pathway leading to nuclear envelope breakdown and mitotic progression in dividing cells [22, 43]. Actually, the growth factor-induced inositol phospholipid breakdown [3, 45] and the growth signals generated through $InsP_3$-induced release of the $[Ca^{2+}]_i$ store [14] provide further evidence for the importance of Ca^{2+} in regulating cell growth and differentiation. Moreover, the recent studies indicated that the Ca^{2+} stores in the ER and the nuclear envelope are connected, TG may also deplete the Ca^{2+} store in the nuclear envelope [34]. The depletion of the nuclear envelope Ca^{2+}

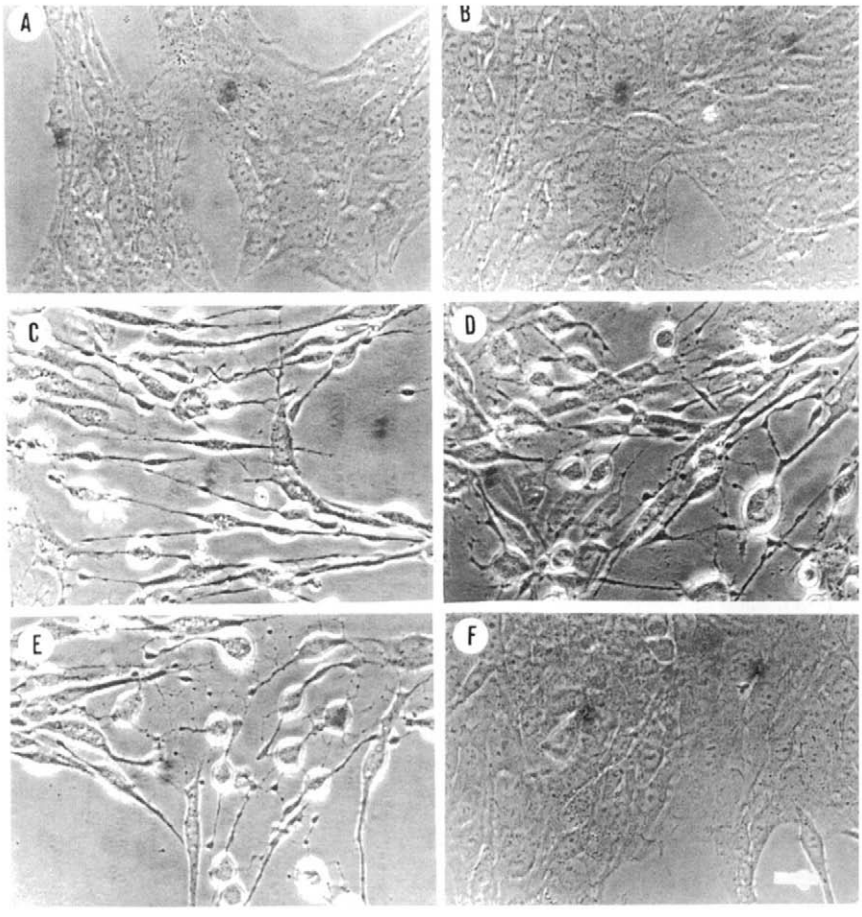

Fig. 6. Changes of cell morphology induced by TG and DBHQ in C6 glioma cells. Note that the morphology of cells changed from a flat round shape (**A**) to a spindle shape after treatment with either 1 μM TG (**C**) or 25 μM DBHQ (**E**) for 48 h. The morphology of control cells was unchanged when cultured for additional 24 h in fresh medium (**B**). The effects of TG were irreversible (**D**) but those of DBHQ (**F**) were reversible by washout after 48 h of treatment followed by a subsequent 24 h culture in drug-free medium. Scale bar = 25 μm.

store can modulate the nuclear pore complexes and prevent movement of medium-sized molecules (10–70 kDa) across the nuclear envelope [24, 33]. Thus the genomic material is sequestered inside the nucleus and the transit of molecules out of the nucleus is disturbed, which could lead to the cell proliferation arrest. Hardingham et al. [15] showed that nuclear and cytoplasmic Ca^{2+} have distinct functions in the control of gene expression. The depletion of the Ca^{2+} store of the ER might influence the activation of transcription factors in controlling the cell cycle processing. The initial increase in the intracellular Ca^{2+} level induced by TG and DBHQ is not involved in proliferation arrest, since we have demonstrated that neither A23187 nor ionomycin could induce proliferation arrest, although they certainly elevated the Ca^{2+} level.

It is well established that CDK inhibitors p21and p27 proteins control the cell cycle arrested at G_1/S transition point [36, 51]. We proceeded to examine the relation between intracellular calcium pool depletion and p21 and p27 production induced by TG and DBHQ. The results showed that intracellular calcium pool depletion led to an increase in p21 and p27 production, and the refilled intracellular calcium store down-regulated p21 and p27 protein expression. Furthermore, the expression of other regulatory proteins controlling G_1/S transition such as, cdc2, CDK2, cyclin C, cyclin D1, cyclin D3 and PCNA were all inhibited by TG and DBHQ and resumed by $[Ca^{2+}]_i$ store refilling. These findings suggest that Ca^{2+} controls cell growth by influencing the expression of cell cycle regulatory proteins (p21 and p27). Since the expression of p21 and p27 proteins is regulated by p53, we examined if TG and DBHQ affected p53 protein expression. The result obtained indicated that C6 glioma cells expressed a high basal level of p53 protein which is a mutant form [52]. The amount of p53 remained unchanged after intracellular calcium store depletion induced by TG and DBHQ. This finding points out that in addition to the known regulators such as p53 [8], serum [27], TGFβ [6] and Myo D [13], the induction of p21 and p27 can also be regulated by the $[Ca^{2+}]_i$ store which is p53-independent [39] in cultured C6 glioma cells.

In this study, we found that TG and DBHQ induced initial proliferation arrest and eventual cell differentiation after 30 h culture. This finding is in accordance with Baranska's report [2] that TG induced transiently increased $[Ca^{2+}]_i$ (even in Ca^{2+}-free buffer) in C6 glial cells with no change in cellular morphology within 15 min to 1 h. Similarly, we did not observe the morphological changes induced by TG unless prolonged incubation for more than 30 h. However, in DDT_1MF-2 smooth muscle cells, both TG and DBHQ arrested the growth and depleted intracellular $InsP_3$-sensitive Ca^{2+} pools, without the morphological changes [11]. It is well known that cAMP-increasing agents or N-substituted cAMP analogues can induce C6 glioma cells to arrest at the G_0G_1 phase and to increase expression of GFAP protein associated with the morphological changes towards a differentiation-typic shape [5, 40, 53]. We wonder if ER Ca^{2+} store depletion may increase cAMP level prior to the proliferation arrest. This possibility is awaiting to be elucidated. On the other hand, when the Ca^{2+} pools refilled, the morphology returned to a prematuration shape and the pattern of GFAP also returned. The results

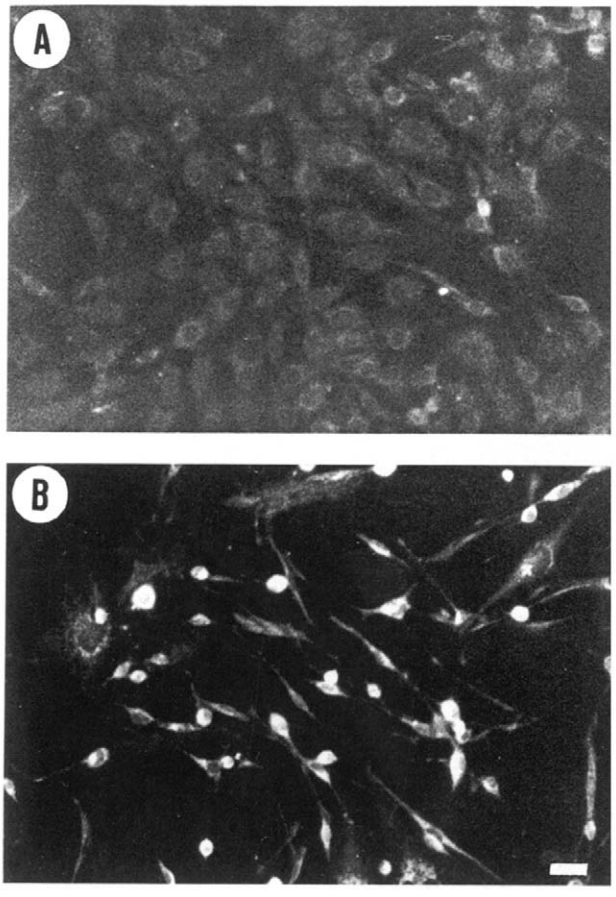

Fig. 7. Staining for glial fibrillary acidic protein (GFAP) in C6 glioma cells. Cells were treated without (**A**) or with (**B**) 1 μM TG for 48 h. Note that TG markedly increased the production of GFAP. Scale bar = 25 μm.

obtained in this study were in agreement with the findings reported by Ghosh et al. [11]. Using the incorporation of [^{3}H]thymidine to evaluate DNA synthesis and cell-cycle phase, they also showed that the cell cycle eventually arrested at the G_0G_1 phase.

It was also reported that TG was capable of inducing apoptosis in many cell lines mediated by depleting the intracellular calcium pool [20, 21, 23], but some cell lines were resistant to TG [11, 28]. In response to stress or damage, the cells increased the expression of wild-type p53 which might increase their sensitivity to stress [1, 26]. In C6 glioma cells, the mutant p53 protein form is persistently expressed at a high level, which may account for the cells' behaviour of not entering apoptosis but remaining at cell growth arrest.

In conclusion, we have demonstrated that TG and DBHQ, two intracellular Ca^{2+} pump inhibitors, not only arrest cell proliferation, but also induce a morphological change typical for the differentiated state. Depletion of the intracellular calcium pool was found to be a link between the induction of CDK inhibitors p21 and p27 proteins, reduction of cell cycle regulatory proteins and cell cycle arrest. These findings suggest that signals generated by TG-sensitive Ca^{2+} pools are important for the control of the cell cycle. Thus, abnormal growth of glioma cells can probably be managed through this growth regulatory pathway.

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