Thallium Acetate Induces C6 Glioma Cell Apoptosis

CHEE-FAH CHIA,[a,b] SOUL-CHIN CHEN,[a] CHIN-SHYANG CHEN,[a] CHUEN-MING SHIH,[b] HORNG-MO LEE,[c] AND CHIH-HSIUNG WU[a]

[a]*Department of Surgery, School of Medicine, Taipei Medical University and Hospital, Taipei, Taiwan*

[b]*Graduate Institute of Medical Sciences, School of Medicine, Taipei Medical University and Hospital, Taipei, Taiwan*

[c]*Graduate Institute of Cell and Molecular Biology, School of Medicine, Taipei Medical University adn Hospital, Taipei, Taiwan*

ABSTRACT: Thallium acetate is a known neurotoxic agent. In this study, we investigated the mechanisms by which thallium acetate induces cell cycle arrest and cell apoptosis in cultured LC6 glioma cells. Exposure of C6 glioma cells to thallium acetate decreased cell viability as demonstrated by the MTT assay. Incubation of thallium acetate arrested cell cycle progression at the G_2/M phase and caused cellular apoptosis at 300 μM as determined by trypan blue exclusion and flow cytometric analysis. The G2/M arrest was associated with a decrease in expression of CDK2 protein and an upregulation of p53 and the CDK inhibitor $p21^{Cip1}$, but not $p27^{Kip1}$. Thallium acetate did not alter the protein levels of cyclin A and B; cyclin D1, D2, and D3; and CDK4 expression in C6 glioma cells. Incubation of C6 glioma cells with thallium acetate upregulated the expression of proapoptotic proteins Bad and Apaf and downregulated the expression of anti-apoptotic proteins Bcl-xL and Bcl-2. In conclusion, these data suggest that thallium acetate inhibits cell cycle progression at G2/M phase by suppressing CDK activity through the p53-mediated induction of the CDK inhibitor $p21^{Cip1}$. Impairment of cell cycle progression may trigger the activation of a mitochondrial pathway and shifts the balance in the Bcl-2 family toward the proapoptotic members, promoting the formation of the apoptosome and, consequently, apoptosis.

KEYWORDS: C6 glioma cells; G_2/M arrest; mitochondria; $p21^{Cip1}$; $p27^{Kip1}$; thallium acetate

INTRODUCTION

Thallium, a toxic heavy metal originally found in coal combustion and cement manufacturing, has become an important environmental pollutant.[1] Thallium salts were once used as medicine to treat tuberculosis, sexually transmitted diseases, and

Address for correspondence: Chih-Hsiung Wu, M.D., Ph.D., Department of Surgery, School of Medicine, Taipei Medical University and Hospital, 252 Wu-Hsing St., Taipei, Taiwan. Voice: +00886-2-27372181 ext. 3311; fax: +00886-2-27369438.
chwu@tmu.edu.tw

Ann. N.Y. Acad. Sci. 1042: 523–530 (2005).
doi: 10.1196/annals.1338.064

ringworm of the scalp.[2] Thallium has also been used as a poison in criminal plots to hurt or kill people. A case of acute thallium poisoning in a suspected murder attempt has been reported in Taiwan recently.[3] The manifestations of thallium toxicity include extremely painful sensory neuropathy, motor paralysis alteration of mental status, and dysfunction and damage of such organs as the heart, liver, and kidney.[4] However, the exact mechanism of thallium toxicity has not been completely elucidated. Thallium interferes with energy production by inhibiting sodium–potassium–ATPase.[5] Thallium-induced brain damage is associated with increases in lipid peroxidation in the brain, suggesting that free radical production plays a pivotal role in thallium toxicity.[6,7] An increase in the generation of reactive oxygen species (ROS) and the disruption of cellular energy production may block cell cycle progression, which in turn may induce cell apoptosis.[8]

Cell cycle progression is controlled by a growing family of cell cycle regulatory proteins, cyclins, and their catalytic subunit cyclin-dependent kinases (CDKs).[9] Arrest of the cell cycle requires a group of CDK inhibitors (CKIs) that interact with the cyclin–CDK complexes and block cell cycle progression.[10] Recent reports have demonstrated that CKI expression is upregulated upon exit from the cell cycle or the maintenance of irreversible growth arrest.[11]

Apoptosis is an endogenous mechanism that regulates cell mass in various organ systems. Apoptosis can be regulated by several Bcl-2 family members. The Bcl-2 family comprises both anti-apoptotic proteins (e.g., Bcl-2 and Bcl-x_L) and proapoptotic proteins (e.g., Bad and Bax).[12] Bcl-2 and Bcl-x_L are mitochondrial membrane proteins that interact with Bax, another mitochondrial membrane-bound proapoptotic protein, to inhibit the Bax homo-oligomeric channel. The proapoptotic protein, Bad, binds and prevents the inhibitory constraint of Bcl-2/Bcl-x_L, resulting in ion flux across the mitochondrial outer membrane and subsequently leading to the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol. Cytochrome *c* binds to the adaptor protein Apaf-1 to promote autoactivation of procaspase-9 and the downstream caspase cascade, causing apoptosis executed by the terminal caspase-3.[13] Apoptosis can be triggered by downregulation of the anti-apoptotic homolog Bcl-2 and Bcl-x_L with or without upregulating the expression of proapoptotic proteins such as Bax and Bak or other apoptosis-related gene products such as p53 and $p21^{Cip1}$.[12]

In this study, we investigated whether thallium acetate regulates cell cycle progression and cell apoptosis in C6 glioma cells. We demonstrated that thallium inhibits DNA replication, leading to cell cycle arrest and cell death. The induction of cell cycle arrest is associated with upregulation of the CDK inhibitor $p21^{Cip-1}$. On the other hand, thallium-induced apoptosis is associated with elevation of proapoptotic proteins, Apaf and Bad, and downregulation of the anti-apoptotic proteins, Bcl-2 and Bcl-x_L.

MATERIALS AND METHODS

Materials

Affinity-purified mouse polyclonal antibodies to cyclin D1, D2, and D3, as well as CDK2, CDK4, $p21^{Cip1}$, and $p27^{Kip1}$, were obtained from Transduction Laboratory (Lexington, KY); Dulbecco's modified Eagle's medium, heat-inactivated fetal

bovine serum, and other reagents required for cell cultures from Life Technologies (Gaithersburg, MD); protease inhibitor cocktail tablets from Boehringer Mannheim (Mannheim, Germany); thallium acetate and other chemicals from Sigma (St. Louis, MO).

Cell Culture, Preparation of Cell Lysates, Polyacrylamide Gel Electrophoresis, and Western Blotting

C6 glioma cells (from Food Industry Research and Development Institute, Hsin-Chu, Taiwan) were cultured as described previously.[14] Cells were lysed by adding lysis buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 0.1% mercaptoethanol, 0.5% Triton X-100, and the protease inhibitor cocktails (final concentrations: 0.2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 50 mg/mL leupeptin). Cells adhering to the plates were scraped using a rubber policeman and stored at –70°C for further measurements. Electrophoresis was carried out using 7.5% sodium dodecyl sulfate–polyacrylamide gel. Following electrophoresis, proteins on the gel were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was incubated with a solution containing appropriate primary antibodies in the blocking buffer. Finally, the PVDF membrane was incubated with peroxidase-linked anti–mouse immunoglobulin G antibodies for 1 h and then developed using a LumiGLO chemiluminescence kit (Amersham, UK).

MTT Assay

The effect of thallium acetate on cell viability was measured by a modified MTT assay, based on the ability of live cells to utilize thiazolyl blue and convert it into dark blue formazan. In brief, 6,000 cells/well (in 96-well microtiter plates) were incubated with different concentrations of thallium acetate in 0.2 mL of culture medium overnight. The supernatant was then removed, and 0.1 mL of 40 mM HCl in isoprotanol was added to each well at 37°C for 2 h. The absorbance at a wavelength of 550 nm was measured with a micro-ELISA reader (Bio-Rad, Hercules, CA). Each assay was performed in triplicate.

Cell Cycle Synchronization and Flow Cytometry

Cells were synchronized at the G_1 phase by serum starvation for 24 h at 37°C. The cells were collected and fixed with 70% ethanol and then washed with phosphate-buffered saline. RNA was lysed with RNAse for 20 min at 37°C and stained with propidium iodide for 2 h at 4°C. The DNA content was measured using a flow cytometry analysis (Becton-Dickinson, San Jose, CA); 15,000 events were analyzed for each sample.

Statistical Analysis

Results are expressed as mean ± standard error of the mean from the number of independent experiments performed. One-way analysis of variance (ANOVA) and Student's two-tailed *t* test were used to determine the statistical significance of the difference between means. A *P* value of less than 0.05 was considered significant.

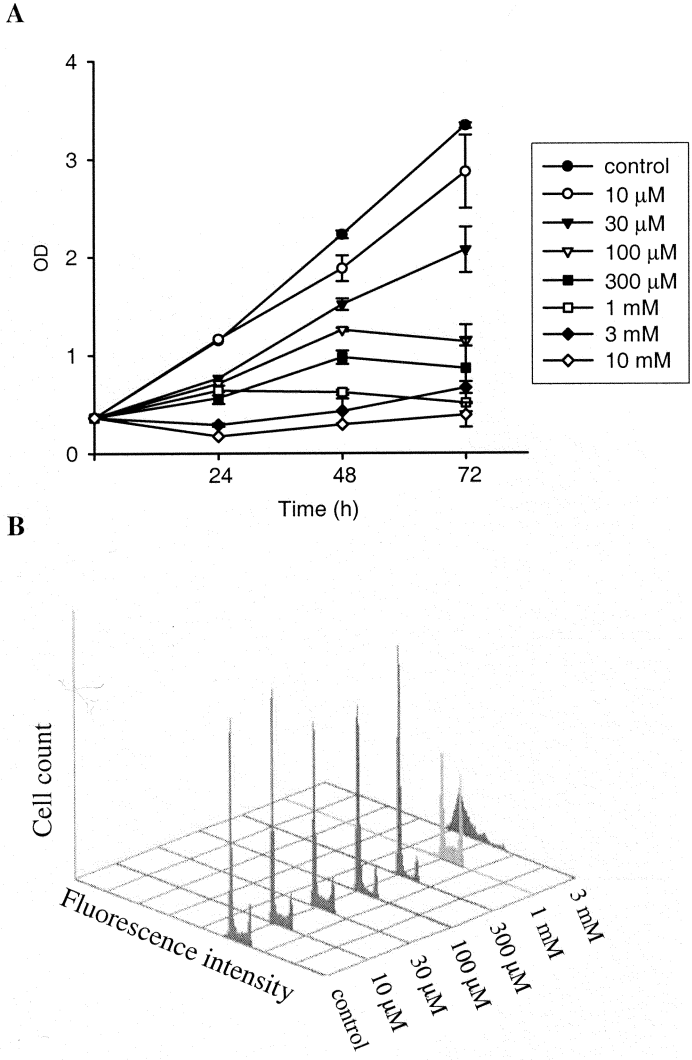

FIGURE 1. Effects of thallium acetate on cell viability and cell cycle progression. (**A**) MTT assay of cell viability revealed a dose- and time-dependent reduction in cell viability following thallium acetate exposure. (**B**) Cell cycle analysis showed an increase in cell population at G_2/M phase after thallium acetate exposure.

RESULTS

Effects of Thallium Acetate on Cell Viability and Cell Cycle Progression

Thallium has been shown to interfere with energy production and increase ROS generation.[6,7] Because oxidative stress is a deleterious event leading to cell death, we investigated whether thallium alters cell viability. C6 glioma cells were incubated with different concentrations of thallium acetate and cell viability determined by the MTT assay. FIGURE 1A shows that cell viability was reduced by thallium acetate treatment in a dose- and time-dependent manner. To further address whether thallium acetate halts cell cycle progression, we synchronized C6 glioma cells by serum starvation for 24 h; serum was then added to the quiescent cells to activate the cell cycle progression. Different concentrations of thallium acetate were added to the growth-stimulated cells for 24 h, and the percentage of the cells in the G_0/G_1 phase and G2/M phase were determined by flow cytometry. As shown in FIGURE 1B, the cell population at G_2/M phase was increased after thallium acetate exposure, suggesting cell cycle arrest at G_2/M phase. Because cell proliferation and death are closely linked to several regulatory proteins, we analyzed the protein levels of cyclins, CDK, and pro- and anti-apoptotic proteins in C6 glioma cells.

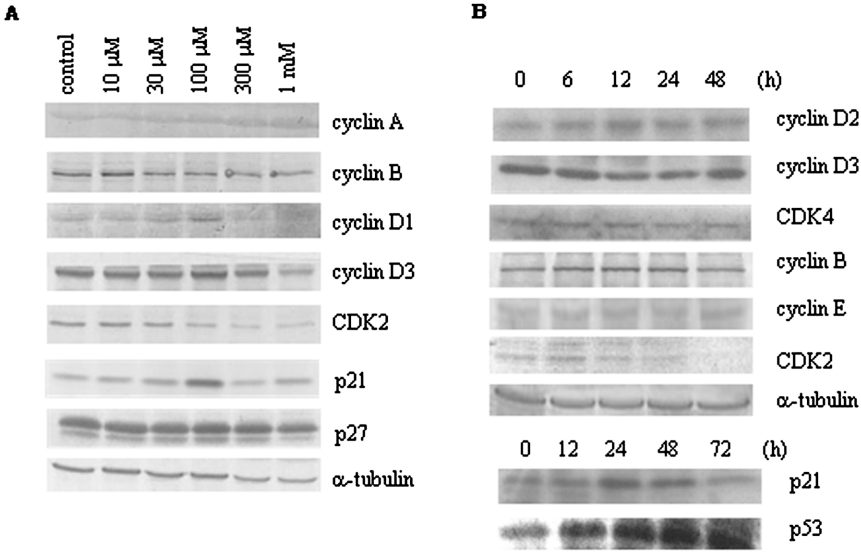

FIGURE 2. Effects of thallium acetate on the expression of cell cycle regulatory proteins. (**A**) Western blot analysis for cyclins and CDKs showed a significant reduction in the protein level of CDK-2 following thallium acetate exposure. (**B**) Western blot analysis revealed that treatment of cells with thallium acetate increased the expressions of $p21^{Cip1}$ and p53.

Effects of Thallium Acetate on Expression of Cell Cycle Regulatory Proteins

To investigate changes in the expression of cell cycle regulatory proteins during these processes, we carried out Western blot analysis for cyclins A, B, D1, D2, D3, E, and CDK2 and CDK4, using specific monoclonal antibodies. CDK2 expression was significantly suppressed by thallium acetate (300 μM) (FIG. 2A). In contrast to CDK2, the expression of CDK4 was not affected. Cell cycle arrest was characterized by increased expression of CDK inhibitors. Treatment of cells with thallium acetate (100 μM) increased the expression of $p21^{Cip1}$ but not $p27^{kip1}$, with the maximum induction of $p21^{Cip1}$ seen after 24 h (FIG. 2B). The induction of $p21^{Cip1}$ was in parallel with an increase in p53 expression.

Effects of Thallium Acetate on Expression of Pro- and Anti-Apoptotic Proteins

The increase of sub-G_1 population (FIG. 1B) suggests that thallium acetate induces C6 glioma cell apoptosis. In an effort to delineate the mechanism, we examined the effect of thallium on the expression of Bcl-2, Bcl-x_L, Bad, and Apaf. Western blot analysis revealed that treatment with thallium acetate for 24 h downregulated the expression of two anti-apoptotic proteins, Bcl-2 and Bcl-x_L (FIG. 3A). Thallium acetate upregulated the expression of proapoptotic proteins Bad and Apaf in C6 glioma cells (FIG. 3B).

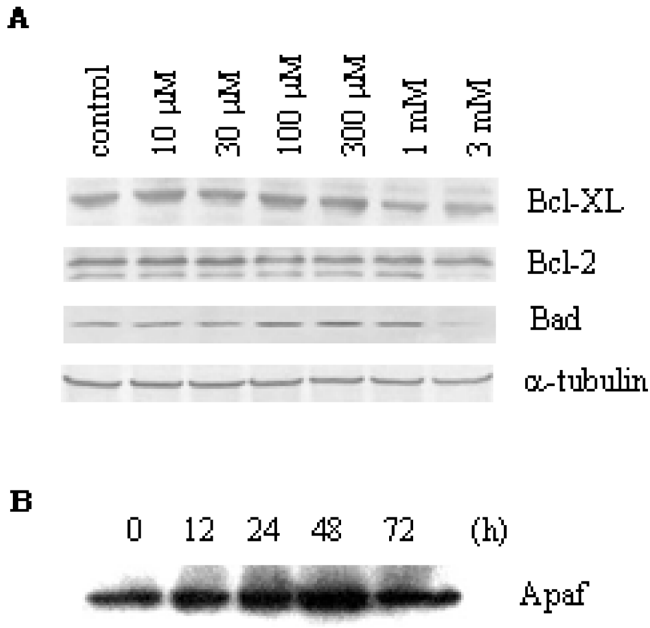

FIGURE 3. Effects of thallium acetate on the expression of apoptosis regulatory proteins. (**A**) Western blot analysis showed that treatment of C6 glioma cells with thallium acetate for 24 h downregulated the expression of anti-apoptotic protein Bcl-2 and Bcl-XL. (**B**) Thallium acetate also upregulated the Bad and Apaf protein levels in C6 glioma cells.

DISCUSSION

Thallium is known to damage the nervous system and organs such as the heart, liver, and kidney. However, little is known regarding the precise mechanisms of thallium toxicity at molecular and cellular levels. This study demonstrated that thallium acetate inhibits cell cycle progression at G_2/M phase by suppressing CDK2 expression and by inhibiting the CDK activity, likely through induction of the CDK inhibitor $p21^{Cip1}$. Impairment of cell cycle progression probably is the mechanism leading to C6 glioma cell apoptosis. Thallium crosses the blood–brain barrier and accumulates in the brain after exposure.[6] Results from this study suggest that thallium exerts its toxic effects in cells in the nervous system via the influence of cell cycle progression and induction of cell apoptosis.

Several mechanisms of thallium toxicities have been proposed. Thallium may substitute for potassium in the Na, K-ATPase pump, possibly due to the similar ionic charges and radii.[5,15] Thallium induces lipid peroxidation, presumably due to an increase in ROS generation.[6] In this study, we demonstrated that thallium toxicity may be associated with induction of cell cycle arrest and apoptosis in C6 glioma cells. Increased ROS generation leads to DNA damage. C6 glioma cells possess normal p53 status,[16] which plays an important role in regulating DNA repair. Induction of p53, probably secondary to ROS-induced DNA damage, may be an initiating event to cause G_1 and G_2 arrest.[17] Thallium acetate increased the expression of both p53 and $p21^{Cip1}$ in C6 glioma cells, suggesting that cell cycle arrest and cell death occurs through a p53-dependent induction of $p21^{Cip1}$. Concomitant with the expression of p53 and $p21^{Cip1}$, downregulation of Bcl-2 and Bcl-x_L (anti-apoptotic proteins) and upregulation of Bad and Apaf, (proapoptotic proteins) were also noted. These apoptosis-regulatory proteins play prominent roles in several cell death paradigms. Bcl-2 and Bcl-x_L suppress, whereas Bad promotes, apoptosis through their actions in preserving and disrupting, respectively, the integrity of the mitochondrial membrane.[18] Downregulation of Bcl-2 and Bcl-x_L and upregulation of Bad may thereby facilitate the disintegration of the mitochondrial membrane, leading to the release of mitochondrial proteins such as cytochrome *c*. Once cytochrome *c* is released from mitochondria into the cytosol, it binds to the adaptor protein Apaf-1 and promotes activation of the caspase cascade to execute the cell death program. Thus, thallium acetate–induced cell cycle arrest at G_2/M followed by the induction of p53 and $p21^{Cip1}$ may be the triggering events. Prolonged cell cycle arrest that resulted in C6 glioma cell apoptosis is likely due to an imbalance in the expression of anti-apoptotic (Bcl-2 and Bcl-x_L) and proapoptotic (Bad) proteins.

A relatively high concentration of thallium acetate is required to induce cell apoptosis. The 50% lethal dose of thallium has been estimated to be 32 mg/kg of body weight in rats (given intraperitoneally).[6] However, because thallium acetate tends to accumulate in rat brain regions,[6] it is difficult to estimate the dose required to exert neurological toxicity *in vivo*.

In summary, we demonstrated that treatment of C6 glioma cells with thallium acetate results in cell cycle arrest and apoptosis induction. Thallium is known to heighten oxidative stress to cause DNA damage. DNA damage may in turn upregulate p53 expression, leading to the induction of CDK inhibitors such as $p21^{Cip1}$. Induction of $p21^{Cip1}$ expression may cause cell cycle arrest and lead to apoptosis.

REFERENCES

1. HOFFMAN, R.S. 2003. Thallium toxicity and the role of Prussian blue in therapy. Toxicol. Rev. **22:** 29–40.
2. HOFFMAN, R.S. 2000. Thallium poisoning during pregnancy: a case report and comprehensive literature review. J. Toxicol. Clin. Toxicol. **38:** 767–775.
3. HUANG, J. *et al*. 1998. Thallium poisoning a clinical analysis of 5 cases. Chin. Med. J. **78:** 610–611.
4. RUSYNIAK, D.E., R.B. FURBEE & M.A. KIRK. 2002. Thallium and arsenic poisoning in a small midwestern town. Ann. Emerg. Med. **39:** 307–311.
5. PEDERSEN, P.A., J.M. NIELSEN, J.H. RASMUSSEN & P.L. JORGENSEN. 1998. Contribution to Tl+, K+, and Na+ binding of Asn776, Ser775, Thr774, Thr772, and Tyr771 in cytoplasmic part of fifth transmembrane segment in alpha-subunit of renal Na, K-ATPase. Biochemistry **37:** 17818–17827.
6. GALVAN-ARZATE, S., A. MARTINEZ, E. MEDINA, *et al*. 2000. Subchronic administration of sublethal doses of thallium to rats: effects on distribution and lipid peroxidation in brain regions. Toxicol. Lett. **116:** 37–43.
7. APPENROTH, D. & K. WINNEFELD. 1999. Is thallium-induced nephrotoxicity in rats connected with riboflavin and/or GSH?—reconsideration of hypotheses on the mechanism of thallium toxicity. J. Appl. Toxicol. **19:** 61–66.
8. ANNUNZIATO, L., S. AMOROSO, A. PANNACCIONE, *et al.* 2003. Apoptosis induced in neuronal cells by oxidative stress: role played by caspases and intracellular calcium ions. Toxicol. Lett. **139:** 125–133.
9. SHERR, C.J. 1995. D-type cyclins. Trends Biochem. Sci. **20:** 187–190.
10. SHERR, C.J. & J.M. ROBERTS. 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. **9:** 1149–1163.
11. HIYAMA, H., A. IAVARONE & S.A. REEVES. 1998. Regulation of cdk inhibitor p21 gene during cell cycle progression is under the control of E2F. Oncogene **16:** 1513–1523.
12. GREEN, D.R. 2003. Overview: apoptotic signaling pathways in the immune system. Immunol. Rev. **193:** 5–9.
13. CREAGH, E.M., H. CONROY & S.J. MARTIN. 2003. Caspase-activation pathways in apoptosis and immunity. Immunol. Rev. **193:** 10–21.
14. LIN, C.H., Y.F. LIN, M.C. CHANG, *et al*. 2001. Advanced glycosylation end products induce nitric oxide synthase expression in C6 glioma cells: involvement of a p38 MAP kinase-dependent mechanism. Life Sci. **69:** 2503–2515.
15. AOYAMA, H., M. YOSHIDA & Y. YAMAMURA. 1986. Acute poisoning by ingestion of thallous malonate. Hum. Toxicol. **5:** 389–392.
16. MOBIO, T.A., E. TAVAN, I. BAUDRIMONT, *et al*. 2003. Comparative study of the toxic effects of fumonisin B1 in rat C6 glioma cells and p53-null mouse embryo fibroblasts. Toxicology **183:** 65–75.
17. TAYLOR, W.R. & G.R. STARK. 2001. Regulation of the G2/M transition by p53. Oncogene **20:** 1803–1815.
18. CHAO, D.T. & S.J. KORSMEYER. 1998. BCL-2 family: regulators of cell death. Annu. Rev. Immunol. **16:** 395–419.