Free Rad. Res., Vol. 35, pp. 1–10
Reprints available directly from the publisher
Photocopying permitted by license only

© 2001 OPA (Overseas Publishers Association) N.V.
Published by license under
the Harwood Academic Publishers imprint,
part of The Gordon and Breach Publishing Group.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/23/11
For personal use only.

Gliotoxin Induces Apoptosis in Cultured Macrophages via Production of Reactive Oxygen Species and Cytochrome c Release without Mitochondrial Depolarization

Y.K. SUEN, K.P. FUNG, C.Y. LEE and S.K. KONG*

Department of Biochemistry, The Chinese University of Hong Kong, Shatin, Hong Kong, China

Accepted by Prof. H. Sies

(Received 7 July 2000; In revised form 8 October 2000)

The cytotoxicity and its underlying mechanisms induced by gliotoxin (GT), an immunosuppressive agent, in macrophages are poorly understood. We report here that GT induced a rapid apoptosis (DNA fragmentation and hypodiploid nuclei obtained within 4 hrs of treatment) in murine macrophages PU5-1.8 in a dose-dependent and cell cycle-independent manner. The GT-induced apoptosis was suppressed by z-Asp, z-VAD-fmk and antioxidants suggesting that production of reactive oxygen species (ROS) and activation of caspases were important in this process. Also, release of cytochrome c from mitochondria was found to be an early event (within 1 hr) after addition of GT (250 ng/ml) and its presence in the cytosol was sufficient to elicit apoptosis. Interestingly, the release of cytochrome c was not accompanied by a reduction in the mitochondrial membrane potential (ψ_m) as determined by several ψ_m-sensitive fluorescent indicators. Taken together, our results indicate that GT is a potent apoptotic agent in PU5-1.8 cells and the loss of ψ_m is not a universal early marker for apoptosis.

Keywords: Gliotoxin, macrophage, apoptosis, ROS, cytochrome c, mitochondrial potential

Abbreviations: GT, gliotoxin; 4OH-TEMPO, 4-hydroxy-2,2,6,6-tetra-methyl-piperidinooxy; MFI, mean fluorescence intensity; ROS, reactive oxygen species; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; z-Asp, z-Asp-CH2-DCB; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

INTRODUCTION

Aspergillosis is a life-threatening infection in human caused by *Aspergillus fumigatus* and *Aspergillus niger*. Now, it is clear that gliotoxin (GT) is the causative agent of these pathogens.[1] GT is known to exhibit a profound suppressive activity in immunocytes including macrophages.[2–6]

*Corresponding author. Fax: 852-2603-5123. E-mail: skkong@cuhk.edu.hk.

RIGHTSLINK

However, the detailed mechanism underlying aspergillosis-induced immunosuppression remains unclear.

One of the interesting features of GT is its ability to go through a redox cycle in the presence of appropriate reducing agents. During redox cycling, the reduced GT generates superoxide anion and during the conversion of the dithiol form of GT to its disulphide form, the superoxide anion is dismutated to hydrogen peroxide,[2] a potent apoptotic stimulus.[7] During apoptosis, one of the common events is the loss of mitochondrial transmembrane potential (ψ_m) and the release of cytochrome c from mitochondria.[8] Once in the cytosol, cytochrome c is competent to activate caspases for DNA fragmentation.[9] Thus, release of cytochrome c from mitochondria is a critical step for caspase activation and cell death. At present, it is unclear whether GT follows this "universal" pathway in macrophages to elicit cell death. To address this question, we examined the cytotoxicity by GT, its capability in releasing reactive oxygen species (ROS) and cytochrome c, and the mode of cell death in a murine macrophage line PU5-1.8 cells.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/23/11
For personal use only.

MATERIALS AND METHODS

Materials and Cell Culture

Dihydroethidium, TMRE, MitoTracker-Red, $DiOC_6(3)$ and JC-1 were purchased from Molecular Probes (Eugene). Other reagents were from Sigma (St. Louis).

Cell Culture, Cell Cycle Analysis and Agarose Electrophoresis for DNA Fragmentation

PU5-1.8 cells, a macrophage cell line derived from Balb/C mice, obtained from American Type Culture Collection (Maryland) were cultured in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco) at 37 °C, 5% CO_2. Cell cycle analysis and DNA fragmentation were performed as previously described.[10,11]

For the Tunel assay or DNA nick labelling, cells after fixation were resuspended in 50 µl reaction buffer (potassium cacodylate, 200 mM; Tris/HCl, 25 mM; BSA, 0.25 mg/ml, pH 6.6) and treated with terminal transferase (5 U/50 µl, Boehringer) and dUTP-FITC (0.2 mM, Boehringer) at 37 °C for 30 min. After washing, cells were treated with RNase A and labelled with propidium iodide (PI). Fluorescence of dUTP-FITC and PI were then measured simultaneously by flow cytometry.

Western Blot Analysis of Cytochrome c Release

PU5-1.8 cells (4×10^6) treated with agents were incubated in lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, 1 mM PMSF, 5 µg/ml leupeptin, 21 µg/ml aprotinin) containing digitonin ($25\,\mu g/4 \times 10^6$ cells) at room temperature for 30 sec to permeabilize the plasma membrane (to damage the mitochondrial membrane required a higher digitonin concentration ($400\,\mu g/4 \times 10^6$ cells)).[11] Supernatants were saved after centrifugation at 10,000 rpm for 1 min. Proteins in the supernatants were then resolved on 12% SDS polyacrylamide gels with the same amount of protein per lane. Proteins so separated were blotted onto PVDF membranes and the amount of cytochrome c released into the cytoplasm was detected by Western blotting and densitometer. The position of cytochrome c in the Western blot was confirmed by a parallel running with an exogenous cytochrome c (data not shown).

Loading Cytochrome c into PU5-1.8 Cells by Electroporation

PU5-1.8 cells (6×10^6) were washed once with PBS and once with permeabilization buffer (in mM: 140 KCl, 5 NaCl, 10 glucose, 0.5 EGTA, 10 HEPES, pH 7.2). The cells were resuspended in 0.3 ml permeabilization buffer containing

RIGHTSLINK

cytochrome c (100 μM) or BSA (100 μM) in an electroporation cuvette. Cells were challenged by a square-wave (voltage: 500 volts, pulse width: 90 msec, number of pulse: 5) with an electroporator (BTX Transfector). The cells were then washed once with 10% FCS and resuspended in medium for further treatment.

Detection of H_2O_2 by Scopoletin and Dihydroethidium

Scopoletin (0.2 μM) was mixed with horseradish peroxidase (1 μg/ml) in PBS. After equilibration, the mixture was transferred to a quartz cuvette and change of fluorescence of scopoletin was measured with a fluorometer (Perkin Elmer LS50B) with a stirring magnetic bar at room temperature. For fluorescence measurement, excitation was made at 350 nm and emission at 460 nm. Known concentration of H_2O_2 was added as a positive control.

The level of intracellular ROS was measured by flow cytometry with dihydroethidium. Briefly, cells (1×10^6/1.5 ml) incubated with dihydroethidium (final concentration 10 μM) at room temperature for 15 min were treated with GT. At various time intervals, cells were submitted to analysis with FACSort (Becton Dickinson). The excitation wavelength was 488 nm and emitted fluorescence was collected at 510 nm. Forward and side scatters were used to establish size gates and exclude debris from the analysis, and the mean fluorescence intensity (MFI) of 10,000 cells within the gates was determined.

Determination of Mitochondrial Membrane Potential (ψ_m)

The ψ_m of cells treated with GT was measured by loading the cells with TMRE (final concentration 500 nM, 15 min), MitoTracker-Red (final concentration 500 nM, 15 min), $DiOC_6(3)$ (final concentration 40 nM, 30 min) or JC-1 (final concentration 10 μM, 10 min) at room temperature. Cells were then submitted to flow cytometric analysis (FACSort, Becton Dickinson). Forward and side scatters were used to establish size gates and exclude debris from the analysis. The excitation wavelength was 488 nm while the emitted signals were collected at the channels for red and green fluorescence. For one single analysis, the fluorescence properties of 10,000 cells within the gates were collected.

RESULTS

Gliotoxin Increased Cellular ROS Content

The ability of GT to increase cellular ROS was examined with dihydroethidium. The working principle of dihydroethidium is that once dihydroethidium is oxidized to ethidium, it inserts into DNA and emits red fluorescence.[12] An increase in red fluorescence therefore represents the amount of ROS released inside the cells. As indicated in Figure 1A, the mean fluorescence intensity (MFI) of ethidium increased gradually with time by using a flow cytometer. The longer the incubation time with GT, the more ROS was released. It is also clear in Figure 1A that the MFI of the GT-treated cells was always higher than that of the untreated group. These observations indicate that GT increased cellular ROS in PU5-1.8 cells.

Next, the ability of GT to generate ROS or H_2O_2 was detected by scopoletin. Scopoletin is a fluorescent compound that can be oxidized by H_2O_2 into a non-fluorescent product in the presence of horseradish peroxidase.[13] As shown in Figure 1B, addition of freshly prepared H_2O_2 (100 nM) to the mixture of scopoletin and horseradish peroxidase led to a decrease in the fluorescence and the drop was more or less the same with the same amount of H_2O_2 added. With this approach, GT was found to decrease the fluorescence of scopoletin until H_2O_2 was removed by catalase (Figure 1B). Together with the previous observations, our results indicate that GT is able to generate ROS by itself in the presence of reducing agents and the

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/23/11
For personal use only.

RIGHTSLINK

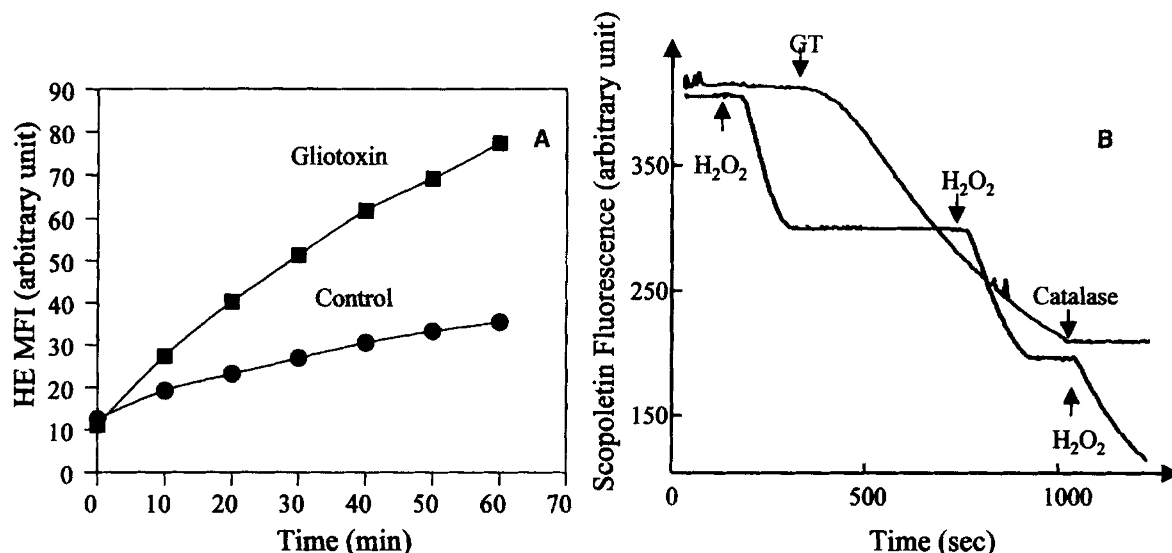

FIGURE 1 Generation of ROS by GT in PU5-1.8 cells (A). Cells (1×10^6/1.5 ml) loaded with dihydroethidium (10 μM) were treated with GT (5 μg/ml) (■) or medium alone (●) at room temperature and the mean fluorescence intensity (MFI) was determined at the time as indicated by flow cytometry. Detection of H_2O_2 by scopoletin in PBS (B). Mixture of scopoletin (0.2 μM) and horseradish peroxidase (1 μg/ml) were prepared with PBS and the change of scopoletin fluorescence was determined by a fluorometer as described in the "Materials and Methods". Freshly prepared H_2O_2 (100 nM), GT (2.5 μg/ml) or catalase (45 U/ml) was added at the time as indicated.

rapid oxidation of dihydroethidium in Figure 1A is largely a result of the generation of H_2O_2 from the GT inside PU5-1.8 cells.

Gliotoxin Induced Apoptosis in PU5-1.8 Cells Through ROS Production

The effect of GT on the induction of apoptosis in PU5-1.8 cells was examined by agarose electrophoresis and flow cytometry. As shown in Figure 2, GT induced apoptosis through DNA fragmentation as indicated by the occurrence of DNA ladders in agarose electrophoresis (Figure 2A) and formation of hypodiploid cells in cell cycle analysis (Figure 2B). Data in Figure 2 also reveal that the action of GT was in a dose- (Figure 2A) and time-dependent (Figure 2C) manner. The number of apoptotic cells increased steadily with time and roughly 43% of the cell population underwent apoptosis within 3 hrs after GT (250 ng/ml) treatment (Figure 2C). From Figure 2B, it seems likely that apoptotic cells were from all phases of cell cycle. This conclusion was supported by the TUNEL assay (DNA nick end labelling by terminal transferase and FITC-conjugated nucleotides) that cells at different cell stages were labelled with dUTP-FITC (Figure 2D). Results in Figure 2B also indicate that apoptosis could not be seen when PU5-1.8 cells were treated with an inactive GT analogue, dimethyl-gliotoxin (2.7 μg/ml) for 5 hrs. Taken together, our results suggest that GT is a specific and cell cycle-independent apoptotic agent in PU5-1.8 cells.

To characterize further the nature of the GT-mediated apoptosis, several antioxidants such as 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy (4OH-TEMPO), propylgallate and 6-hydroxy -2,5, 7,8-tetramethylchroman-2-carboxylic acid (trolox) were added to the culture medium, and their effect on the GT apoptosis was determined.[14] It is clear in Figure 3A that 4OH-TEMPO inhibited the GT (250 ng/ml)-elicited DNA fragmentation in a dose-dependent manner. Flow cytometric analysis also shows that 4OH-TEMPO (20 mM), propylgallate (1 mM) and trolox

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/23/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/23/11
For personal use only.

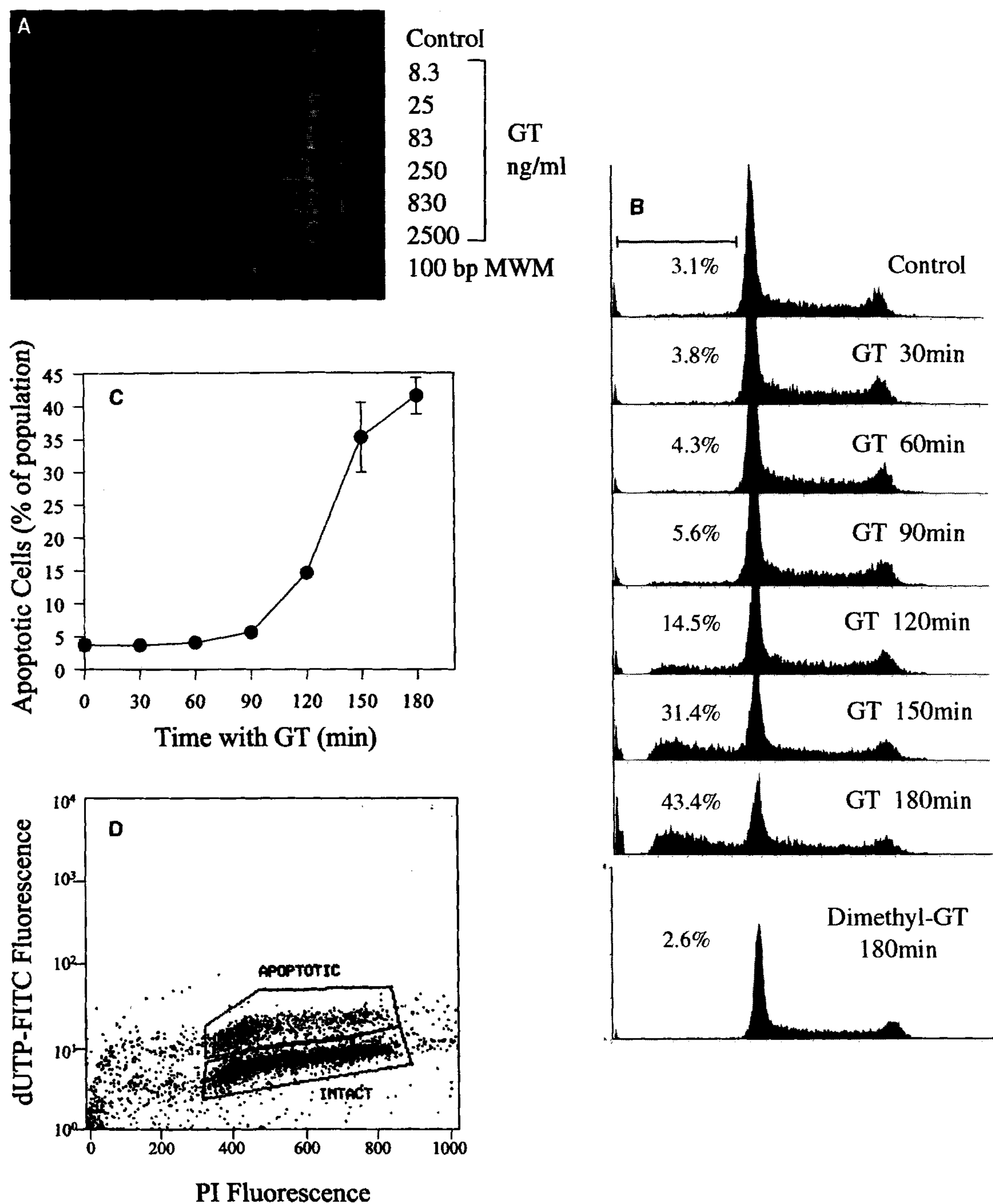

FIGURE 2 Effect of GT on the induction of apoptosis in PU5-1.8 cells. (A) DNA from cells (1×10^6/1.5 ml) treated with the concentration of GT as indicated at 37 °C for 4 hrs was extracted and analysed by agarose gel electrophoresis. (B) PU5-1.8 cells (1×10^6/1.5 ml) were treated with GT (250 ng/ml), dimethyl-GT (2.7 μg/ml) or medium alone at 37 °C for the time as indicated. Cells were then fixed and stained with propidium iodide (PI). DNA content was subsequently determined by flow cytometry. (C) Percentage of hypodiploid cells in (B) was measured and plotted against time. Results are mean of duplicate determinations. (D) PU5-1.8 cells (1×10^6/1.5 ml) were treated with GT (250 ng/ml) at 37 °C for 4 hrs. After fixation, DNA nicks in the cells were labelled with dUTP-FITC and terminal transferase. Cells were then stained with PI and the fluorescence of FITC and PI determined simultaneously.

RIGHTSLINK

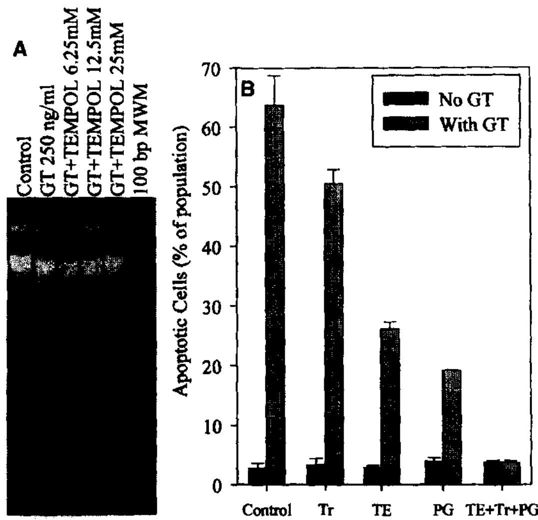

FIGURE 3 Effect of antioxidants on the GT-mediated apoptosis. (A) DNA from cells (1×10^6/1.5 ml) treated with GT (250 ng/ml) at 37 °C for 4 hrs in the presence of 4OH-TEMPO at the concentration as indicated was extracted and analysed by agarose gel electrophoresis. (B) PU5-1.8 cells (1×10^6/1.5 ml) pre-treated with 4OH-TEMPO (TE, 20 mM), trolox (Tr, 3 mM), propylgallate (PG) (1 mM) or a combination of the three antioxidants for 15 min were treated with or without GT (250 ng/ml) at 37 °C for 4 hrs. After fixation, cells were stained with propidium iodide and the percentage of hypodiploid cells was calculated by flow cytometry. Results are mean ± SD of triplicate determinations.

(3 mM) produced different degree of protection against the apoptotic effect of GT (Figure 3B). Under our experimental conditions, propylgallate was more potent than 4OH-TEMPO and trolox provided the least protection. If cells were pretreated with all three antioxidants, almost all the apoptotic effect was removed (Figure 3B). Collectively, our results indicate that the apoptosis induced by GT is due to, at least in part, the production of ROS in PU5-1.8 cells.

Gliotoxin Released Cytochrome c from PU5-1.8 Cells without a Concomitant Drop of Mitochondrial Potential

Intrigued by the understanding that mitochondria play a key role in apoptotic pathways,[8] we examined the role of mitochondria in the GT-mediated apoptosis. To study cytochrome c release from mitochondria, cytosolic extracts from the cells treated with GT were carefully prepared to keep mitochondria intact.[11] As can be seen in Figure 4A, cytochrome c was detected in the cytosol within 60 min of the treatment with GT (250 ng/ml) and its level increased steadily throughout the experiment (Figure 4A). It is generally believed that cytochrome c, in conjunction with caspase-9, can activate caspase-3.[9] To evaluate this, PU5-1.8 cells were treated with z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, an inhibitor of caspase-3 and several other caspases)[15] or z-Asp (z-Asp-CH2-DCB, a general caspase inhibitor)[16] in the presence of GT. At a final concentration of 25 μM, z-VAD-fmk prevented the apoptosis by GT (Figure 4B). Similar observation was obtained with z-Asp (100 μM) (Figure 4B). To further confirm the role of cytochrome c in the induction of apoptosis in PU5-1.8 cells, we introduced exogenous cytochrome c (100 μM) or BSA (100 μM) into PU5-1.8 cells by electroporation and the change of apoptosis was studied. As shown in Figure 4C, cytochrome c induced apoptosis in PU5-1.8 cells while BSA did not elicit any effect. Our results thus indicate that release of cytochrome c from mitochondria after GT treatment is an important step in eliciting apoptosis in PU5-1.8 cells.

Reduction in mitochondrial membrane potential (ψ_m) has been implicated in the release of cytochrome c and apoptotic process.[8] To investigate further the effect of GT on the mitochondria, we tested whether GT treatment elicited a decrease in ψ_m in PU5-1.8 cells using TMRE, a ψ_m-specific fluorescent indicator.[17] As can be seen in Figure 5A, the fluorescence of TMRE was able to show depolarized mitochondria when cells were treated with CCCP, a protonophore that completely dissipates ψ_m in minutes.[18] Interestingly, treating cells with GT (250 ng/ml) for 150 min did not alter the ψ_m (Figure 5A). When the incubation time was extended to 180 min, a positively skewed pattern, without

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/23/11
For personal use only.

RIGHTS LINK

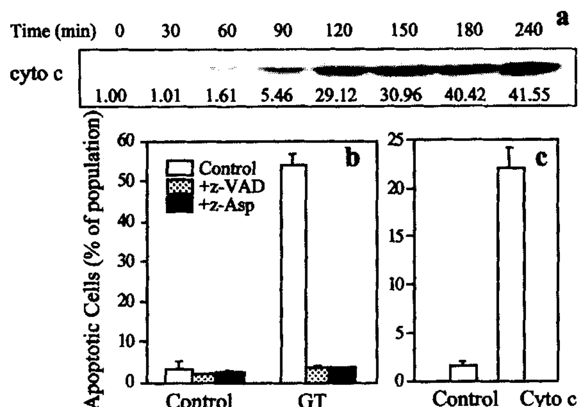

FIGURE 4 Effect of GT on the cytochrome c release and caspase activation. (A) cytochrome c (cyto c) in the cytosol of PU5-1.8 cells treated with GT (250 ng/ml) at 37°C for various time intervals was detected by Western blotting. The protein bands of 15 kDa were scanned and the relative density was shown individually below the protein bands. (B) PU5-1.8 cells (1×10^6/1.5 ml) treated with GT (250 ng/ml) in the absence or presence of z-VAD-fmk (25 µM) or z-ASP (100 µM) for 4 hrs at 37°C were subject to cell cycle analysis by flow cytometry and the percentage of hypodiploid cells was then determined. (C) Effect of cytochrome c on the induction of apoptosis. PU5-1.8 cells were loaded with cytochrome c (100 µM) or BSA (100 µM) by electroporation. Cells were then incubated in culture medium with 10% FCS at 37°C for 2 hrs. Cell population with hypo-diploid DNA was then determined by flow cytometry. Results are mean ± SD of triplicate determinations.

much change in the mode, was observed when compared to control (Figure 5A). These results suggest that a loss of ψ_m does not appear to be a concomitant event with the release of cytochrome c. Similar conclusion was obtained with other ψ_m-sensitive fluorescent indicators such as MitoTracker-Red[19] (Figure 5B) or $DiOC_6(3)$[20] (Figure 5C).

In view of the fact that JC-1 is a more reliable probe for analyzing ψ_m changes with flow cytometry,[21] we therefore examined the effect of GT again on ψ_m with JC-1. JC-1 exists in monomeric form and emits green fluorescence after excitation at 488 nm. In polarized mitochondria, JC-1 undergoes aggregate formation and emits red fluorescence. As shown in Figure 5D, treating cells with GT (250 ng/ml) for 150 min did not change the ψ_m as compared to that of GT-untreated cells. Similar to our previous observation, the peak of the population shifted to the right in terms of JC-1 green fluorescence when the incubation time was increased to 180 min. In the presence of CCCP, most of the JC-1 red fluorescence disappeared (Figure 5D). These results therefore indicate that GT could not reduce the ψ_m in PU5-1.8 cells.

DISCUSSION

In our study, fungal GT was found to be a strong apoptotic agent in murine cultured macrophages PU5-1.8. DNA fragmentation and hypodiploid cells formation were observed 4 hrs after the addition of GT (250 ng/ml). Consistent with these observations, cytochrome c was detected in the cytosol within 1 hr of treatment. Moreover, apoptotsis elicited by exogenous cytochrome c after electroporation implies that cytochrome c in the cytosol is sufficient to execute apoptotic pathway. In the GT-treated cells, a reduction in the degree of apoptosis caused by antioxidants and caspase inhibitors suggests that release of ROS and activation of caspases are important events in this apoptotic pathway. In fact, addition of freshly prepared H_2O_2 was able to release cytochrome c in PU5-1.8 cells and GT could rapidly induce membrane blebbings and DNA fragmentation in human and mouse peritoneal macrophages (data not shown). Results in our study therefore explain why GT is a potent immunosuppressive agent.

Recently, several hypotheses have been proposed to explain the release of cytochrome c from mitochondria. The best known one involves the opening of the mitochondrial permeability transition pores (MPTP). With the opening of MPTP, water and solutes influx into the mitochondrial matrix, leading to mitochondrial depolarization, swelling, rupture of the outer mitochondrial membrane and release of cytochrome c.[22] The second model involves a voltage dependent anion channel (VDAC) on the outer mitochondrial membrane. According to

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/23/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/23/11
For personal use only.

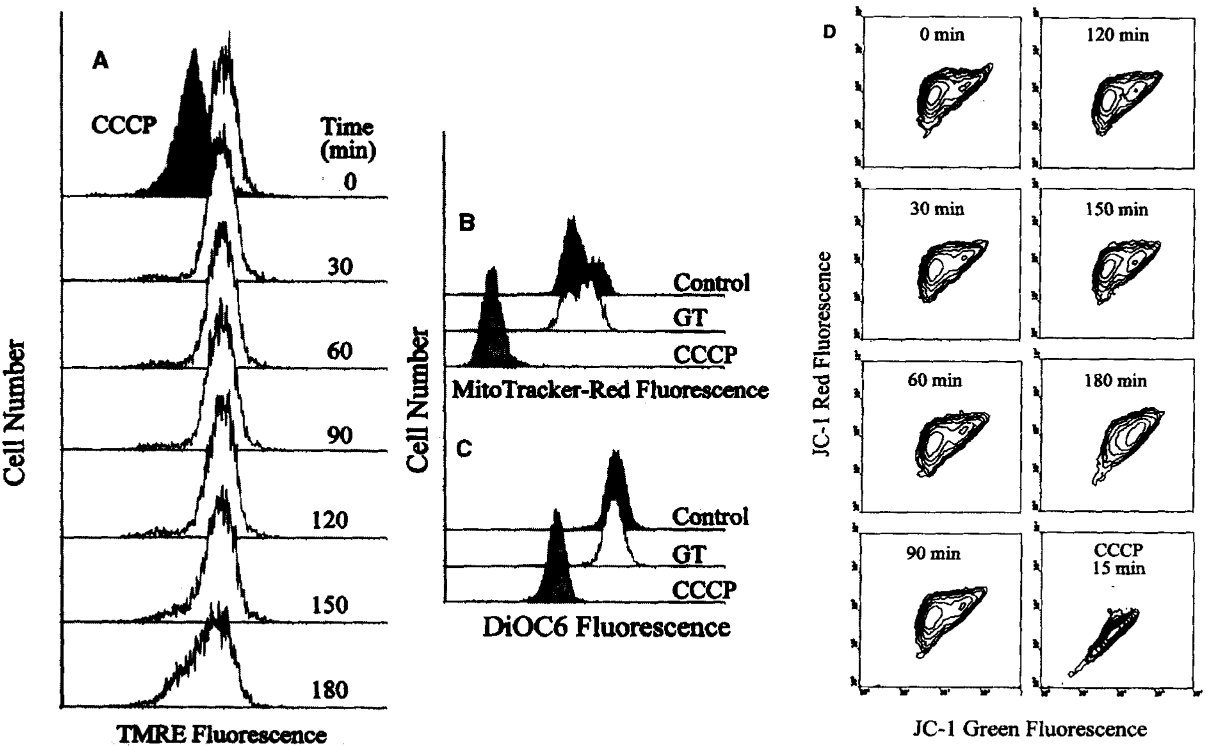

FIGURE 5 Measurement of mitochondrial transmembrane potential (ψ_m) in GT-treated PU5-1.8 cells with different ψ_m-sensitive fluorescent indicators. PU5-1.8 cells (1×10^6/1.5 ml) treated with or without GT (250 ng/ml) at 37 °C for the time as indicated were loaded with TMRE (500 nM) for 15 min (A), MitoTracker-Red (500 nM) for 15 min (B), $DiOC_6(3)$ (40 nM) for 30 min (C) or JC-1 (10 mM) for 10 min at room temperature and the ψ_m was assessed by flow cytometry. Depolarized ψ_m was obtained by treating cells with CCCP (70 μM) as a positive control. Data shown here are from a single experiment representative of at least two independent experiments.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/23/11
For personal use only.

the VDAC model, closure of VDAC during apoptosis leads to a defect in mitochondrial ATP/ADP exchange, mitochondrial hyperpolarization, rupture of the outer membrane and leakage of cytochrome c.[23] Both models involve mitochondrial damages and changes in ψ_m. Examples can be found in LLC-PK1 cells in which GT caused cytochrome c release and ψ_m dissipation.[24] In contrast to these observations, a significant amount of cytochrome c was detected in the cytosol of PU5-1.8 cells treated with GT (250 ng/ml, 3 hrs) (Figure 4A) while the ψ_m was normal (Figure 5). Under this condition, ~43% of the population were hypodiploid cells (Figure 2B). Therefore, it is very likely that release of cytochrome c was not accompanied by a loss of ψ_m in the GT-mediated apoptosis. This conclusion was confirmed with several ψ_m-sensitive fluorescent indicators including TMRE, MitoTracker-Red, $DiOC_6$ and JC-1 (Figure 5). In fact, similar independence of ψ_m depolarization has been reported in the apoptotic process induced by a number of death stimuli.[25–27] At present, the mechanism for the release of cytochrome c from mitochondria in the absence of collapse of ψ_m is still unclear. It is tempting to speculate that there might be two pools of cytochrome c in the mitochondria. A major pool in the intermembrane space is available for release while the other pool in the electron transport chain is to maintain mitochondrial respiration and polarization during GT-mediated apoptosis.

Acknowledgements

This project was partially supported by a grant (CUHK 4060/97 M) from Research Grants Council of Hong Kong.

References

[1] R.J. Manuel and C.C. Kibbler (1998) The epidemiology and prevention of invasive aspergillosis. *Journal of Hospital Infection*, **39**, 95–109.

[2] P. Waring and J. Beaver (1996) Gliotoxin and related epipolythiodioxopiperazines. *General Pharmacology*, **27**, 1311–1316.

[3] P. Sutton, N.R. Newcombe, P. Waring and A. Mullbacher (1994) *In vivo* immunosuppressive activity of gliotoxin, a metabolite produced by human pathogenic fungi. *Infection and Immunity*, **62**, 1192–1198.

[4] A. Yamada, T. Kataoka and K. Nagai (2000) The fungal metabolite gliotoxin: immunosuppressive activity on CTL-mediated cytotoxicity. *Immunology Letters*, **71**, 27–32.

[5] L.S. Yoshida, S. Abe and S. Tsunawaki (2000) Fungal Gliotoxin Targets the Onset of Superoxide-Generating NADPH Oxidase of Human Neutrophils. *Biochemical and Biophysical Research Communications*, **268**, 716–723.

[6] P. Waring (1990) DNA fragmentation induced in macrophages by gliotoxin does not require protein synthesis and is preceded by raised inositol triphosphate levels. *Biological Chemistry*, **265**, 14476–14480.

[7] T. Matsura, M. Kai, Y. Fujii, H. Ito and K. Yamada (1999) Hydrogen peroxide-induced apoptosis in HL-60 cells requires caspase-3 activation. *Free Radical Research*, **30**, 73–83.

[8] G. Kroemer, B. Dallaporta and M. Resche-Rigon (1998) The mitochondrial death/life regulator in apoptosis and necrosis. *Annual Review of Physiology*, **60**, 619–642.

[9] I. Budihardjo, H. Oliver, M. Lutter, X. Luo and X. Wang (1999) Biochemical pathways of caspase activation during apoptosis. *Annual Review of Cell and Developmental Biology*, **15**, 269–290.

[10] Samuel Ko, T.T. Kwok, K.P. Fung, Y.M. Choy, C.Y. Lee and S.K. Kong (2000) Slow rise of Ca^{2+} and slow release of reactive oxygen species are two cross-talked events important in tumour necrosis factor-α-mediated apoptosis. *Free Radical Research*, **33**, 295–304.

[11] Y.K. Suen, K.P. Fung, Y.M. Choy, C.Y. Lee, C.W. Chan and S.K. Kong (2000) Concanavalin A induced Apoptosis in Murine Macrophage PU5-1.8 Cells through Clustering of Mitochondria and Release of Cytochrome c. *Apoptosis*, **5**, 369–377.

[12] N. Zamzami, P. Marchetti, M. Castedo, D. Decaudin, A. Macho, T. Hirsch, S.A. Susin, P.X. Petit, B. Mignotte and G. Kroemer (1995) Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *Journal of Experimental Medicine*, **182**, 367–377.

[13] K. Staniek and H. Nohl (1999) H_2O_2 detection from intact mitochondria as a measure for one-electron reduction of dioxygen requires a non-invasive assay system. *Biochimica Et Biophysica Acta*, **1413**, 70–80.

[14] Q. Chen, M. Galleano and A.I. Cederbaum (1997) Cytotoxicity and apoptosis produced by arachidonic acid in Hep G2 cells overexpressing human cytochrome P4502E1. *Journal Biological Chemistry*, **272**, 14532–14541.

[15] M. Yabuki, K. Tsutsui, A.A. Horton, T. Yoshioka and K. Utsumi (2000) Caspase activation and cytochrome c release during HL-60 cell apoptosis induced by a nitric oxide donor. *Free Radical Research*, **32**, 507–514.

[16] T. Sasagawa, M. Hlaing and T. Akaike (2000) Synergistic Induction of Apoptosis in Murine Hepatoma Hepa 1-6 Cells by IFN-gamma and TNF-alpha. *Biochemical and Biophysical Research Communications*, **272**, 674–680.

[17] G.R. Budinger, J. Duranteau, N.S. Chandel and P.T. Schumacker (1998) Hibernation during hypoxia in

RIGHTSLINK

cardiomyocytes. Role of mitochondria as the O_2 sensor. *Journal Biological Chemistry*, **273**, 3320–3326.
[18] T. Minamikawa, D.A. Williams, D.N. Bowser and P. Nagley (1999) Mitochondrial permeability transition and swelling can occur reversibly without inducing cell death in intact human cells. *Experimental Cell Research*, **246**, 26–37.
[19] M. Poot, L.L. Gibson and V.L. Singer (1997) Detection of apoptosis in live cells by MitoTracker red CMXRos and SYTO dye flow cytometry, *Cytometry*, **27**, 358–364.
[20] S. Matsuyama, J. Llopis, Q.L. Deveraux, R.Y. Tsien and J.C. Reed (2000) Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nature Cell Biology*, **2**, 318–325.
[21] S. Salvioli, A. Ardizzoni, C. Franceschi and A. Cossarizza (1997) JC-1, but not $DiOC_6(3)$ or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. *FEBS Letter*, **411**, 77–82.
[22] I. Marzo, C. Brenner, N. Zamzami, J.M. Jurgensmeier, S.A. Susin, H.L. Vieira, M.C. Prevost, Z. Xie, S. Matsuyama, J.C. Reed and G. Kroemer (1998) Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science*, **281**, 2027–2031.
[23] M.G. Vander Heiden, N.S. Chandel, X.X. Li, P.T. Schumacker, M. Colombini and C.B. Thompson (2000) Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival. *Proceedings of National Academy Sciences of the USA*, **97**, 4666–4671.
[24] X. Zhou, A. Zhao, G. Goping and P. Hirszel (2000) Gliotoxin-induced cytotoxicity proceeds via apoptosis and is mediated by caspases and reactive oxygen species in LLC-PK1 cells. *Toxicological Sciences*, **54**, 194–202.
[25] E. Bossy-Wetzel, D.D. Newmeyer and D.R. Green (1988) Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO Journal*, **17**, 37–49.
[26] D.M. Finucane, N.J. Waterhouse, G.P. Amarante-Mendes, T.G. Cotter and D.R. Green (1999) Collapse of the inner mitochondrial transmembrane potential is not required for apoptosis of HL60 cells. *Experimental Cell Research*, **251**, 166–174.
[27] A.J. Krohn, T. Wahlbrink and J.H. Prehn (1999) Mitochondrial depolarization is not required for neuronal apoptosis. *Journal of Neuroscience*, **19**, 7394–7404.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/23/11
For personal use only.

RIGHTSLINK