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Gliotoxin Enhances Radiotherapy Via Inhibition of Radiation-Induced GADD45a, p38, and NFκB Activation

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Abstract The purpose of the study was to elucidate the mechanism underlying the enhancement of radiosensitivity to ^{60}Co γ-irradiation in human hepatoma cell line HepG2 pretreated with gliotoxin. Enhancement of radiotherapy by gliotoxin was investigated in vitro with human hepatoma HepG2 cell line. Apoptosis related proteins were evaluated by Western blotting. Annexin V/PI and reactive oxygen species (ROS) were quantified by Flow Cytometric (FACS) analysis. Gliotoxin (200 ng/ml) combined with radiation (4 Gy) treated cells induced apoptosis. Cells treated with gliotoxin (200 ng/ml) prior to irradiation at 4 Gy induced the expression of bax and nitric oxide (NO). The gliotoxin-irradiated cells also increased caspase-3 activation and ROS. Gadd45a, p38, and nuclear factor kappa B (NFκB) activated in irradiated cells was inhibited by Gliotoxin. Specific inhibitors of p38 kinase, SB203580, significantly inhibited NFκB activation and increased the cytotoxicity effect in cells exposed to gliotoxin combined with irradiation. However, SB203580 did not suppress the activation of Gadd45a in irradiated cells. Gliotoxin inhibited anti-apoptotic signal pathway involving the activation of Gadd45a-p38-NFκB mediated survival pathway that prevent radiation-induced cell death. Therefore, gliotoxin, blocking inflammation pathway and enhancing irradiation-induced apoptosis, is a promising agent to increase the radiotherapy of tumor cells. J. Cell. Biochem. 104: 2174–2184, 2008.

Key words: apoptosis; radiotherapy; gliotoxin; Gadd45a; p38

Surgery and chemical treatments have been generally used as the methods of cancer therapy. Unfortunately, most of hepatoma is not curable because extensive resection is not possible. Though many approaches, such as transarterial chemoembolization, percutaneous ethanol injection, radiofrequency ablation, radiotherapy, and liver transplantation have been developed for the treatment of them, a large number of patients would suffer from recurrence and metastasis [Hanazaki et al., 2000; Takayama et al., 2000]. In order to improve the overall therapeutic effects of liver cancer, the therapies combined with several treatments have been attempted. The purpose of combined therapies for hepatoma is to increase their therapeutic efficiencies and to reduce the side effects and complications. Radiotherapy can be used under the situation where operation is difficult. The radioresistance of tumor cells to radiotherapy-induced cell death is a major problem that limits the successful treatment of human cancer. Among the different molecular strategies that contribute to the resistance of tumor cells to radiotherapy, the role played by NFκB has been well defined. Treatment of cells with radiation and some anti-cancer drug generally lead to the activation of NFκB through a phosphorylation on serines 32 and 36 of I-κBα. When cells induce an activation of NFκB, an apoptosis is blocked by the inhibition of caspase-3 activation. Caspase proteases are the key mediators of an apoptosis, which in turn is regulated by antiapoptotic proteins belonging to the inhibitor of an apoptosis (IAP) family [Li et al., 1998; Holcik et al., 2000]. Thereby, a constitutive activation of NFκB induces overexpression of its

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downstream targets such as bcl-xL, bcl-2, vascular endothelial growth factor, and interleukin-8, which may mediate a resistance to an apoptosis induced by chemotherapy and radiation [Barkett and Gilmore, 1999; Dubner et al., 1995]. A number of in vitro studies have recently shown that the production of inflammatory mediators is strongly affected by mitogen-activated protein kinases (MAPKs) such as p38 MAPK, c-Jun NH2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) [Kumar et al., 1998; Scherle et al., 1998]. A recent study has suggested that p38 MAPK contributes to chemokine transcription by modulating p65 NFκB-mediated transactivation [Schmeck et al., 2004].

The Gadd45 family of genes has been known to be rapidly induced by a variety of genotoxic stresses as well as by terminal differentiation and apoptotic cytokines in nearly all mammalian cells [Fornace et al., 1992; Zhan et al., 1994]. Interestingly, recent reports have suggested that Gadd45 proteins also function in cell survival. It has been shown that Gadd45a and Gadd45b cooperate to promote cell survival via distinct signaling pathway involving activation of the Gadd45a-p38-NFκB-mediated survival pathway in hematopoetic cells exposed to UV radiation [Gupta et al., 2006].

Gliotoxin is a highly toxic second metabolite produced by diverse species of fungi such as *Gliocladium*, *Aspergillus*, and *Penicillium* [Richard et al., 1994]. Gliotoxin has been classified to the epipolythiodioxopiperazine (ETP) group of fungal metabolites and known to possess antibacterial, antiviral, and immunosuppressive activities [Sutton et al., 1994; Waring and Beaver, 1996; Waring et al., 1998]. Recently, the metabolite has been reported to inhibit the activation of NFκB in different cell lines by preventing the degradation of IκBα [Pahl et al., 1996; Kroll et al., 1999]. Furthermore, gliotoxin has enhanced the radio sensitivity of HL-60 cells [Baust et al., 2003]. However, the effect of gliotoxin on radiation-induced p38-NFκB-mediated survival pathway is not explained.

In this study, we examined the induction of an apoptosis in human hepatoma cells by a combination of radiation and gliotoxin, and then the protective effect of gliotoxin on the inflammation by an irradiation. In addition, we analyzed the possible role of p38 MAPK and Gadd45a on the upstream of NFκB in this process.

MATERIALS AND METHODS

Reagents and Antibodies

Gliotoxin and SB203580 were purchased from Sigma Chemical Company (St. Louis, MO). Annexin V-fluorescein isothiocyanate was obtained from BD Biosciences (San Diego, CA). Polyvinylidene difluoride membrane was purchased from Bio-Rad. Antibodies against Gadd45, Bax, NFκB p50, phosphor-IκBα, and caspase-3 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against p38 and phospho-p38 were obtained from Cell Signaling Technologies. All other chemicals were commercially available products of analytical grade.

Cell Culture

HepG2 human hepatoma cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in a Dulbecco's minimum essential medium (DMEM) (Gibco) supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air.

Gliotoxin Treatment and Irradiation

Gliotoxin was purchased from Sigma and stored at −20°C. Gliotoxin stock solutions were prepared in 5 mg/ml DMSO and diluted in DMEM medium prior to use. Exponentially growing HepG2 cells were incubated with 50–250 ng/ml gliotoxin for 2 h prior to irradiation with 4 or 8 Gy of γ-radiation.

Determination of Cell Viability

To evaluate the cytotoxicity of gliotoxin and irradiation, a 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine cell viability. Cells were seeded in 24-well plates at a density of 4×10^4 cells/well and treated with gliotoxin and irradiation. After the treatment, the medium was removed and the cells were washed with phosphate-buffered saline (PBS). Fresh medium was added and the cells were incubated with 100 μl of 1 mg/ml MTT for 3 h. The number of viable cells was directly proportional to the production of formazan, which was solubilized in isopropanol and measured spectrophotometrically at 570 nm.

DNA Fragmentation

Apoptosis was confirmed by detecting the fragmentation of chromosomal DNA using the classic DNA ladder method. Briefly, 2×10^6 cells were immersed in nuclear lysis buffer (0.5% Triton X-100, 20 mM EDTA, and 5 mM Tris; pH 8.0) with 200 μg/ml RNase A and incubated at 37°C for 30 min. The mixture was centrifuged at 12,000*g* at 4°C for 30 min. Chromosomal DNA was extracted using the phenol–chloroform method and precipitated using ethanol. After washing in cold 70% ethanol, the resulting DNA pellet was resuspended in 40 μl of Tris–EDTA buffer (pH 7.5). The extracted DNA was analyzed by electrophoresis on 2.0% agarose gel.

Measurement of Apoptosis by Annexin V Staining

Cells were seeded onto six-well plates at 4×10^4 cells/well, pretreated with 200 ng/ml gliotoxin for 2 h, and then exposed to radiation at 4 Gy. The cells were typsinized and gently washed with serum-containing culture medium followed by PBS. The cells were resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, 25 mM $CaCl_2$) and incubated with annexin V-FITC and propidium iodide (PI; MBL, Japan) at room temperature for 15 min. Fluorescence analysis was carried out using a flow cytometer (Beckman FC500). The signals from annexin V-FITC were detected using the FL1 detector and the PI signals were detected using the FL3 detector.

Protein Extraction and Western Blot Analysis

After gliotoxin treatment, the medium was removed, and the cells were rinsed twice with PBS. After the addition of 0.6 ml of cold RIPA buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.5% Na-deoxycholate [w/v], 0.1% SDS [w/v], and 1% Triton × 100 [w/v]) and the protease inhibitor cocktail supplied by Sigma. The cells were scraped at 4°C. The cell lysate was then centrifuged at 10,000*g* at 4°C for 10 min. The obtained proteins were separated by electrophoresis on 12% polyacrylamide gel and transferred onto nitrocellulose membranes. The membranes were stained with ponceau to confirm the uniform transfer of all samples and then incubated in a blocking solution of PBS with 0.05% Tween-20 (PBST) and 5% non-fat powdered milk at room temperature for 1 h. The membranes were reacted with the following antibodies: caspase-3, Gadd45a, iNOS, and bax (Santa Cruz, CA) at a dilution of 1:1,000 for 90 min, followed by extensive washes with PBST. The membranes were then incubated with 1:1,000 horseradish peroxidase-conjugated secondary antibodies (Zymed) for 1 h, washed with PBST, and developed using the ECL kit.

Measurement of ROS Using DCFH-DA

The intracellular generation of ROS was measured using carboxy-H_2DCF-DA, which is a cell-permeable dye. Inside the cells, this compound is oxidized by ROS to form a fluorescent carboxydichlorofluorescein (DCF). Briefly, cells seeded in six-well plates at 2×10^4 cells/well and treated with or without gliotoxin were incubated with 5 μM carboxy-H_2DCF-DA at 37°C for 15 min. The cells were then washed twice with PBS, trypsinized, and resuspended in OptiMem I medium. The rate of oxidation of the dye in the cells was monitored by measuring the fluorescence with a flow cytometer (Beckman Coulter FC500), using an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

Measurement of Nitrite Concentration

Cells were cultured in 24-well plates in 1 ml of culture medium until confluence. They were treated with gliotoxin in the exposure or without exposure of ionizing radiation at 4 Gy for 72 h, and then the culture media were collected. Accumulated nitrite, an oxidative product of NO, was measured in the culture medium by Griess reaction. Briefly, 100 μl of cell culture medium were mixed with 100 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydorchloride/2.5% phosphoric acid) and incubated at room temperature for 10 min, and then the absorbance at 550 nm was measured in a microplate reader.

Statistical Analysis

All experiments were performed in triplicate and the results are expressed as the mean ± standard deviation. Statistical significance was analyzed using one-way analysis of variance (ANOVA), and the differences among means were determined using Duncan's multi-range tests. $P \leq 0.05$ was considered statistically significant (STATSTICA 2.0).

RESULTS

Gliotoxin showed to induce the HepG2 cell death in a concentration-dependent manner. In

the range of doses used (0–250 ng/ml), the cell viability rate was apparently reduced. The irradiation of HepG2 cells also caused a dose-dependent inhibition for cell growth. The percentages of the cell viability were decreased by $82.3 \pm 3.6\%$ and $77.0 \pm 1.3\%$ at 72 h after an irradiation of 4 and 8 Gy, respectively, which are the usual doses for a clinical treatment (Fig. 1A). Pretreatment of the HepG2 cells with gliotoxin markedly increased the radiation-induced cell death in a time-dependent manner (Fig. 1B). LD_{50} was evaluated to be 200 ng/ml of gliotoxin at 4 Gy. These Results indicate that the combination of gliotoxin and radiation was approximately 2.5 times more effective than radiation alone for the HepG2 cell death rate.

After 72 h of a gliotoxin treatment with or without radiation (4 Gy), cells death rate was assessed with a flow cytometry by a double staining with annexin V and PI. The annexin V-FITC-positive population of the cells (apoptotic cells; lower right quadrant) was not increased in the control cells. However, the apoptotic cell population was increased to 27.3%, and also the annexin V-FITC-positive/PI-positive population (necrosis and late apoptotic cells; upper right quadrant) was increased to 12.3% by radiation in gliotoxin pretreated cells (Fig. 2A). As shown in Figure 2B, a treatment of 0.2 μg/ml gliotoxin prior to radiation (4 Gy) induced a DNA fragmentation after the incubation for 72 h, but gliotoxin alone did not induce a DNA fragmentation (Fig. 2B).

We examined the inhibitory effect of gliotoxin for a phosphorylation on IκBα. Irradiation to the HepG2 cell without gliotoxin, as shown in Figure 3A, resulted in both an expression of NFκB p50 protein and a phosphorylation on IκBα. However, the gliotoxin treated HepG2 cells prior to an irradiation did not show any signals for the expression and phosphorylation, indicating a remarkable inhibition effect for the radiation-induced phosphorylation of IκBα protein.

Based on the fact that caspase-3 activation is initiated when apoptosis occurs, the enzyme activation in response to gliotoxin with radiation was examined by using the immunoblotting technique. Cell lysates, prepared as described above, were proved with a specific antibody to determine the expression changes of caspase-3 proenzymes as indicators for caspase-3 activation. Figure 3B shows that caspase-3 was slightly activated at 24 h after a 0.2 μg/ml gliotoxin treatment. But the caspase-3 activation was clearly detected after the exposure to gliotoxin in combination with radiation after 12 h. The levels of bax protein were elevated in the irradiated HepG2 cells after 24 h (Fig. 3B). On the other hand, the levels of Bax were elevated at an early stage after the exposure to gliotoxin in combination with radiation. These results suggest that the expression of irradiation-induced apoptosis was very weakly expressed, but gliotoxin in combination with radiation-induced Bax protein induction and caspase-3 were consistent with a series of programmed cell death events.

DCF-DA is a widely used fluorescence probe, frequently being used to evaluate the redox state of a cell. Although DCF-DA is used to measure the concentration of hydrogen peroxide in cells, the generated superoxide, nitric oxide, and lipid peroxide are also capable of oxidizing 2′-7′-dichlorodihydrogluorescein. DCF fluorescence in gliotoxin or radiation treated cells was slightly

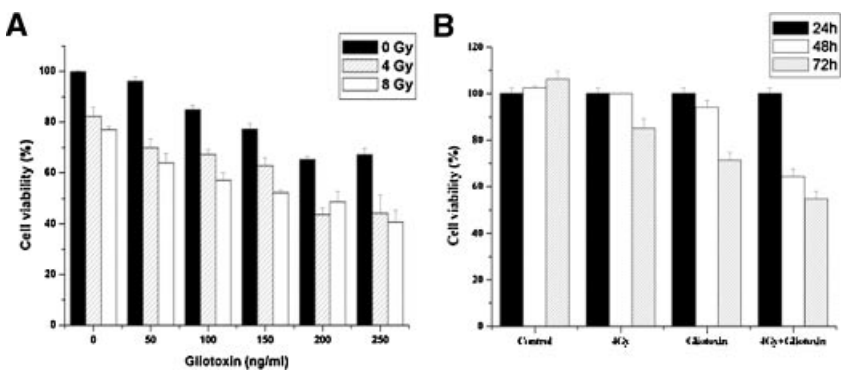

Fig. 1. Combined effect of gliotoxin and radiation on cell viability in HepG2 cells. Cells were incubated with 50–250 ng/ml of gliotoxin and then exposed to irradiation with 4 and 8 Gy (**A**). HepG2 cells were treated by gliotoxin, radiation, or the combination of gliotoxin-radiation for 24, 48, and 72 h (**B**). The percentage of cell viability was determined by MTT assay after 72 h. The control cell was considered to be 100% viable.

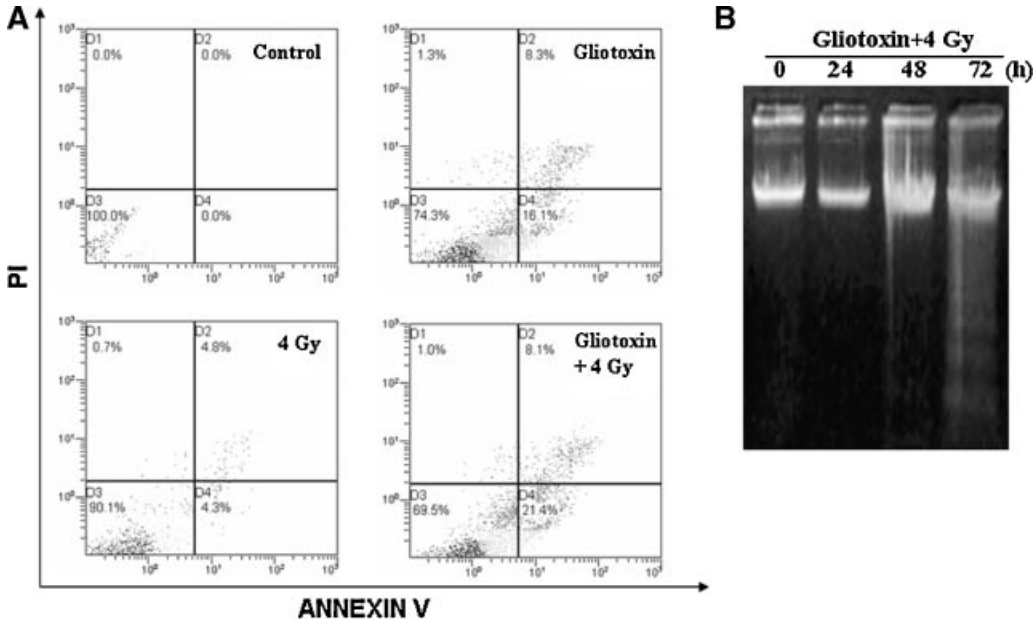

Fig. 2. Apoptosis induction on HepG2 cells by gliotoxin and radiation. Cells were incubated with 200 ng/ml of gliotoxin and irradiated with 4 Gy. **A**: Assessment of cell death was carried out by a flow cytometry. Early apoptotic cells, which are the annexin V-FITC-positive/PI-negative population of cells, are reported in the lower right quadrant. Necrosis or late apoptotic cells, which are the annexin V-FITC-positive/PI-positive population of cells, are reported in the upper right quadrant. **B**: The cells were pretreated with gliotoxin (200 ng/ml for 2 h) and were irradiated (0 and 4 Gy) by a time-dependent manner. DNA fragmentation was measured by 2.0% agarose gel electrophoresis.

higher than that in the non-treated cells, but markedly increased in the cells pretreated by gliotoxin combined with radiation (Fig. 4A). The ROS value of the cells treated by gliotoxin for 2 h and by the radiation was determined to be 20.985 ± 0.0495 and 26.625 ± 1.845, respectively. However the gliotoxin-pretreated cells combined with the radiation showed the ROS value of 62.230 ± 0.071 (Fig. 4B).

The Bcl-2 family of proteins plays a central role in the regulation of apoptotic cell death induced by a wide variety of stimuli such as NO. To evaluate whether NO was related with cell death by gliotoxin or irradiation, the concentration of nitrite was investigated in culture media after the incubation for 72 h. In the gliotoxin pretreated cells combined with the radiation, the production of NO was more significantly increased than in the cells treated by gliotoxin alone (Fig. 5A). To elucidate the increasing mechanism of NO, we further investigated their effects on iNOS expression by Western blot analysis. The expression level of iNOS induced by the radiation was clearly decreased after 12 h in the irradiated cells. However, the radiation combined with gliotoxin showed an apparently increased expression of iNOS (Fig. 5B). The expression of p53 was activated by gliotoxin combined with/without irradiation, but not activated by irradiation alone (Fig. 5C).

To determine whether γ-radiation could induce a phosphorylation of p38 in HepG2 cells or not, Western blot analysis was performed using a phosphor-specific p38 antibody as described in Materials and Methods Section. Figure 6A shows that an increased phosphorylation of p38 was observed in the irradiated HepG2 cells for 90 min, but not in those of gliotoxin treated prior to irradiation. This result clearly indicated that the radiation-induced phosphorylation of p38 was inhibited by gliotoxin treatment before radiation. The pretreatment with p38 inhibitor, SB203580, did not change the reduced viability of 4 Gy-irradiated cells, but the combined pretreatment with SB203580 and gliotoxin significantly improved the reduced viability of irradiated cells (Fig. 6B).

Gadd45a play a survival role against pro-apoptotic stress in hematopoietic cells. The expression of Gadd45a was investigated to understand the related role of gliotoxin. Gadd45a was induced in the irradiated cells,

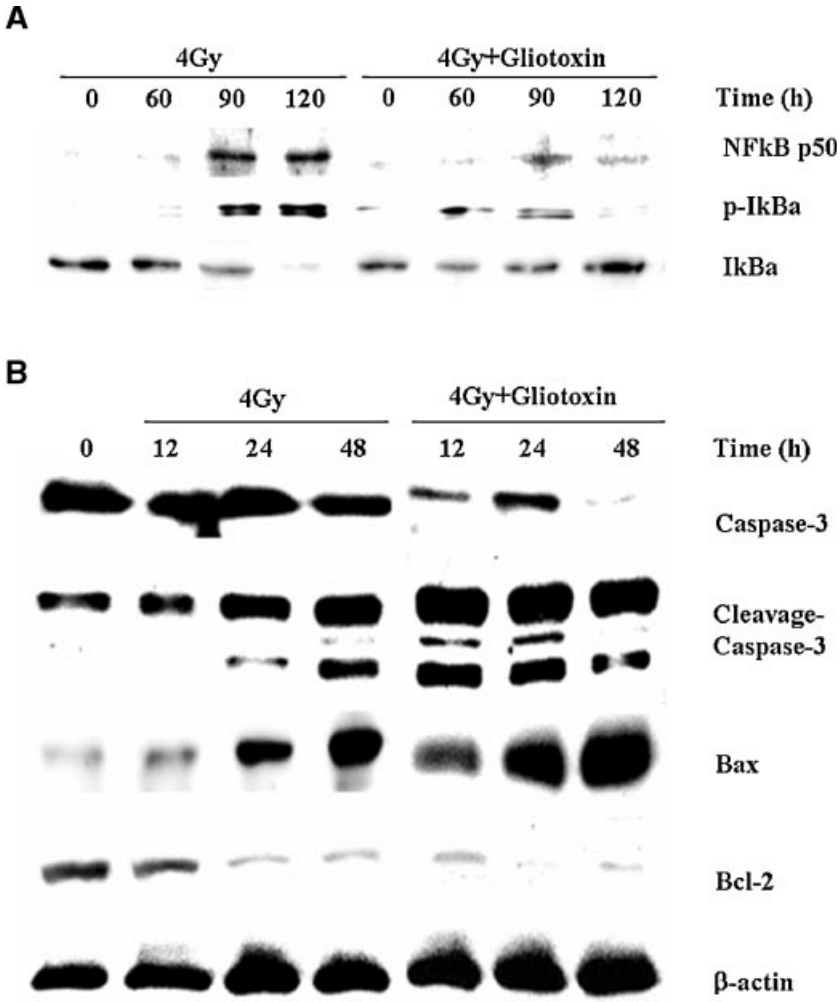

Fig. 3. **A**: Effect of gliotoxin on irradiation-induced NFκB activation in HepG2 cells. Cells were pretreated with gliotoxin for 2 h, followed by irradiation. The cells lysate levels of p50 and IκBα were determined by Western blotting. **B**: HepG2 cells were pretreated with gliotoxin for 2 h and then combined with or without irradiation and examined over time. Changes in caspase-3, Bax, and Bcl-2 protein levels were detected using immunoblotting.

but the pretreatment with gliotoxin inhibited the induction of Gadd45a in the irradiated cells (Fig. 7A). However, the expression of Gadd45a in the irradiated cells was not inhibited by SB203580. Also, the pretreatment with SB203580 blocked the radiation-induced NFκB activation (Fig. 7B).

DISCUSSION

Gliotoxin has emerged as apoptosis lipid effectors through its mitochondrial-interacting and NFκB suppressing function [Elsharkawy et al., 1999]. Based on this dual function, gliotoxin may actually modulate cancer resistance, a phenomenon characterized by the activation of pathways that circumvent the toxicity of current cancer therapies. It has previously shown that gliotoxin enhances radiotherapy in HL-60 [Baust et al., 2003]. The enhancement of radiation-induced apoptosis by gliotoxin showed an additive effect according to the model of Steel and Peckham [1979]. However, the relevance of these signaling pathways and the precise mechanism of activation appear to be different depending on the cellular context and the dose of gliotoxin and irradiation, and it has not been fully characterized. Therefore, we have determined the enhancement of radiotherapy by gliotoxin, in the human hepatoma HepG2 cell line. Our result indicated that cells were treated with gliotoxin prior to

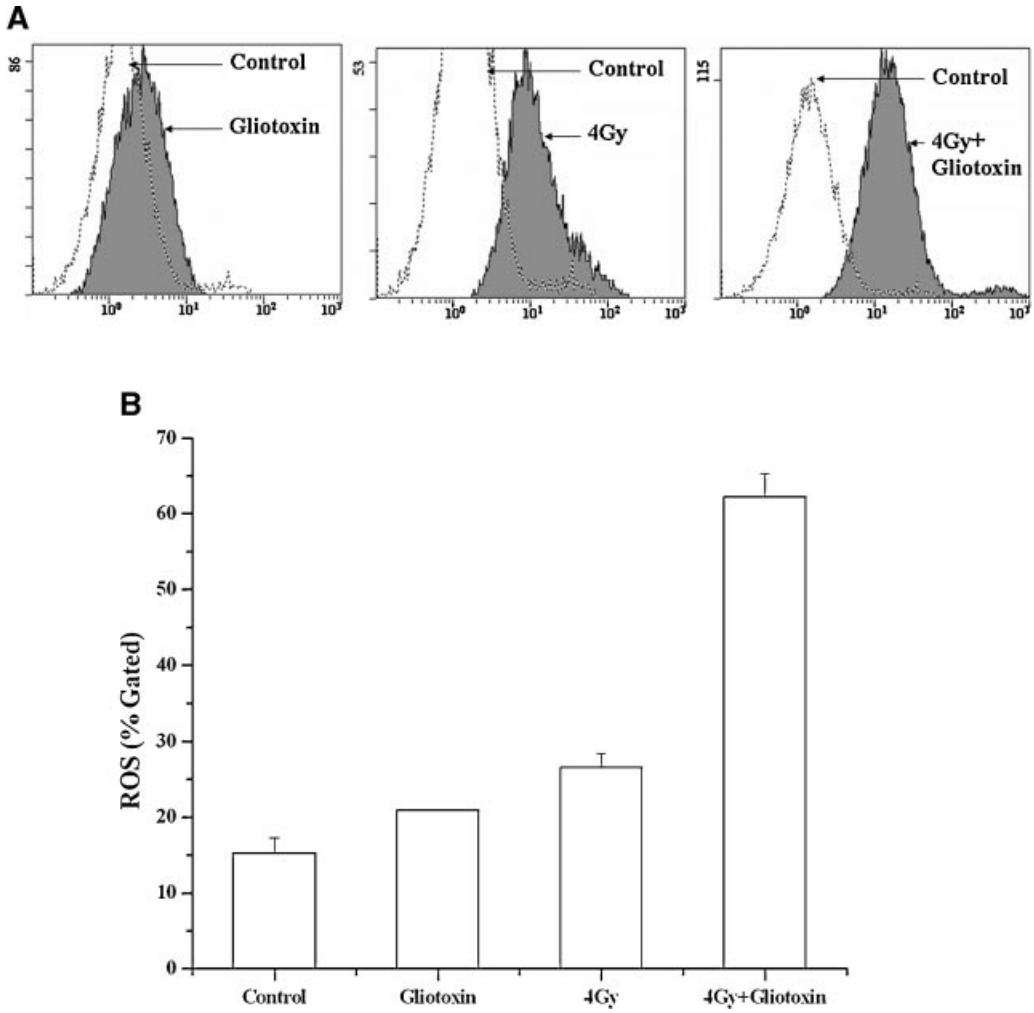

Fig. 4. Generation of reactive oxygen species (ROS) in HepG2 cells treated with 200 ng/ml gliotoxin for 2 h, followed by without or with 4 Gy of radiation. **A**: Flow cytometry assay of ROS generation. **B**: The ratio of gated ROS generation. Values are expressed as the mean standard deviation of three independent experiments.

irradiation; the fraction of apoptotic cells significantly was increased by the release of Bax and activation of caspase-3.

A pivotal mechanism leading to the apoptosis and enhancing radio-sensitivity is considered to be the inhibition of the transcription factor NFκB, which induces the expression of anti-apoptotic genes [Shao et al., 1997; Wang et al., 1998]. The activation of NFκB is normally achieved by the phosphorylation of its main inhibitor, IκBα, at two serine sites (Ser-32 and Ser-36) by IκB kinases [Baldwin, 1996]. The levels of IκBα and phospho-I κBα were not changed and the NFκB DNA-binding activity detected by EMSA was only slightly increased by irradiation with 6 Gy in HL-60 cells [Baust et al., 2003]. However, in this study it showed that the levels of NFκB p50 and phospho-IκBα were increased by irradiation with 4 Gy in HepG2 cells. Gliotoxin has been reported to selectively inhibit NFκB [Elsharkawy et al., 1999]. In certain cell models, gliotoxin can block the activation of NFκB via the inhibition of IκBα degradation by the proteasome [Pahl et al., 1996]. Likewise, the present data shows that gliotoxin can suppress the activation of NFκB by irradiation.

It is well known that ROS play pivotal roles in DNA damage and apoptosis [Lin et al., 2003] and in cell death induced by irradiation [Szotowski et al., 2007]. In this study, gliotoxin or irradiation alone slightly increased ROS, but their combination increased ROS to higher levels. Therefore, the gliotoxin is considered to enhance radiotherapy via the generation of ROS in HepG2 cells.

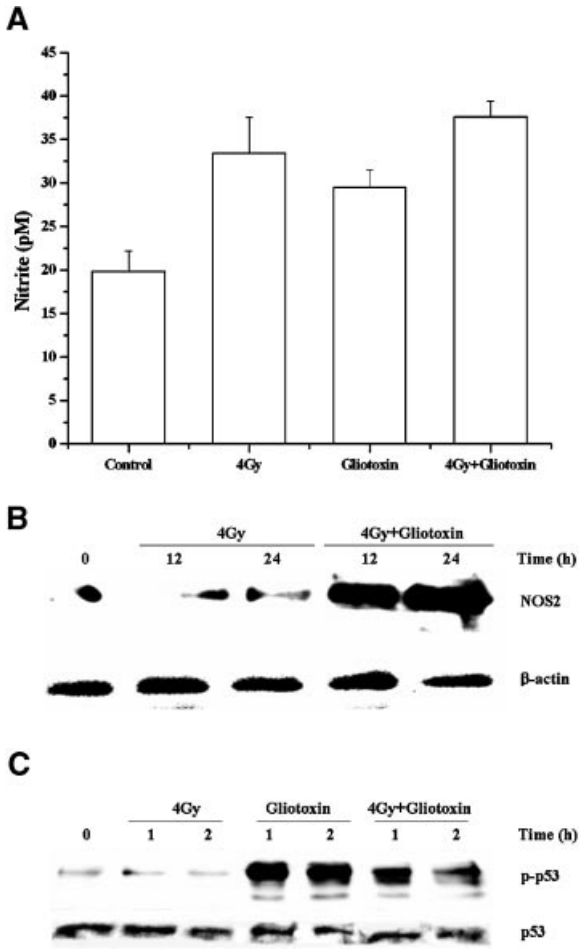

Fig. 5. Effects of gliotoxin combined with or without radiation on the induction of nitric oxide (NO) in HepG2 cells. **A**: Concentration of NO in the cell culture medium was measured. Western blots of iNOS (**B**) and p53 (**C**) expression. The cells were pretreated with gliotoxin for 2 h and then treated with radiation (4 Gy).

High concentrations of NO or peroxynitrite induce cell death, if not by apoptosis, then by necrosis. However, NO induces biochemical characteristics of apoptosis in several cell types through the activation of apoptotic signaling cascade such as caspases [Kim et al., 2000] and the mitochondrial cytochrome *c* release [Brown and Borutaite, 1999], the regulation of cell survival, or the expression of apoptotic gene [Kim et al., 1997; Tamatani et al., 1998; Kwak et al., 2000]. In this study, the level of NO was more increased in HepG2 cells received by both gliotoxin and radiation than in those treated by gliotoxin or radiation alone, which also indicates the enhancement of radiotherapy by gliotoxin via the induction of NO in the cells.

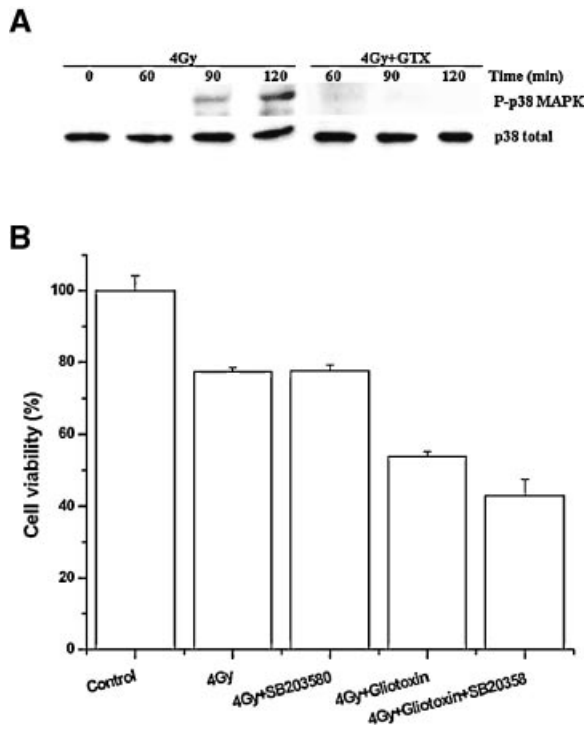

Fig. 6. Effects of gliotoxin on irradiation-induced p38 MAPK activation in HepG2 cells. Cells were pretreated with gliotoxin for 2 h, followed by irradiation. **A**: The cells lysate levels of phosphorylation of p38 and p38 were determined by Western blotting. **B**: Affection of p38 MAPK inhibitor on cell viability of 4 Gy-irradiated HepG2 cells. The cells pretreated with SB203580 and gliotoxin were exposed to 4 Gy. The cells viabilities were detected after 72 h using MTT assay.

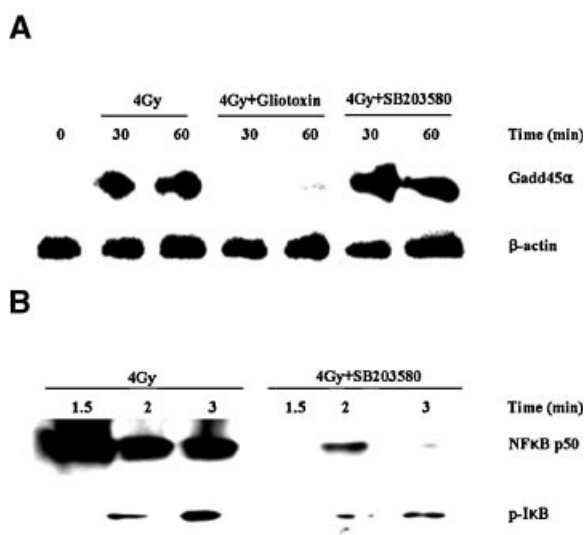

Fig. 7. **A**: Expression of Gadd45 protein in HepG2 cells irradiated after pretreated with or without gliotoxin. The cell lysates used to measure p38 activities were examined at the indicated times for Gadd45a protein expression by Western blot, probing with a polyclonal antibody against Gadd45a. **B**: Effects of p38 MAPK inhibition on irradiation-induced NFκB activation.

The expression of the tumor suppressed gene, p53, is linked to apoptosis in tumor cells that are exposed to DNA damaging agents. Cytotoxic effects of NO and peroxynitrite on tumor cells are the result of DNA damage [Tamir et al., 1996; Cai et al., 2000]. DNA damage induced by NO results in the accumulation of p53, which has been described as an essential indicator of NO-mediated apoptosis [Messmer et al., 1996]. NO-mediated p53 accumulation has been reported to induce cell cycle arrest through p21 up-regulation, or apoptosis through Bax up-regulation [Kolb, 2000]. In the present study, the level of NO was increased, but the expression of p53 was not shown in the cells treated with only irradiation. On the contrary, the increase of NO production and the expression of p53 were observed in the gliotoxin-pretreated cells combined with or without irradiation. These results imply that the generation of NO in the irradiated cells is related to cell survival in preference to apoptotic pathway, and the NO production in the cells treated with gliotoxin proceeds apoptosis.

Gadd45a and *Gadd45b* protect myeloid heatopoietic cells from genotoxic stress-induced apoptosis [Gupta et al., 2005]. Previous reports have shown that *Gadd45b*, but not *Gadd45a*, mediates the activation of p38 by TGFβ which promotes hepatocyte cell death [Yoo et al., 2003]. On the other hand, *Gadd45a* was identified as a target for p53 function [Kastan et al., 1992; Selvakumaran et al., 1994; Guillouf et al., 1995]. It has been well known that Gadd45a regulates cell cycle. Furthermore GADD45a-mediated activation of p38 and NFκB has been reported to play a major role in cell survival [Karin and Lin, 2002; Papa et al., 2004]. In addition, GADD45a-mediated activation of p38 results in the phosphorylation and degradation of IκB, which in turn allows nuclear localization of NFκB and the activation of its target genes [Gupta et al., 2005]. In this study, the activation of p38 and GADD45a was induced by gamma irradiation. However, cell death in SB203580 (p38 inhibitor) treated cells was more increased than in the cells treated by gliotoxin plus irradiation. Interestingly, we found that the activation of p38 and Gadd45a were suppressed in HepG2 cells treated with gliotoxin prior to irradiation.

In conclusion, we have shown that the cell survival in human hepatoma HepG2 cells exposed to gamma radiation is promoted via the activation of the Gadd45a-p38-NFκB and gliotoxin enhances radiotherapy by suppressing the expression of Gadd45a before NFκB activation (Fig. 8). Therefore, our present data suggest that gliotoxin enhances radio-sensitizing via the inhibition of Gadd45a-p38-NFκB mediated signal pathway in human hepatoma HepG2 cells. In addition, we provide mechanistic evidences that gliotoxin enhanced apoptosis in irradiated human hepatoma cells by increasing the expression of p53, generation of ROS, and activation of Bax and caspase-3.

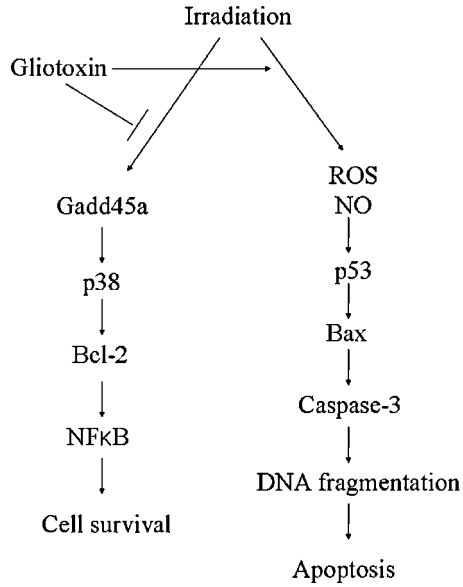

Fig. 8. Schematic diagram indicating that gliotoxin enhances radiotherapy by inhibition of Gadd45a-mediated activation of a p38-NFBsurvial pathway in irradiated HepG2 cells.

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REFERENCES

Baldwin AS Jr. 1996. The NF-kappa B and I kappa B proteins: New discoveries and insights. Annu Rev Immunol 14:649–683.

Barkett M, Gilmore TD. 1999. Control of apoptosis by Rel/NF-κB transcription factors. Oncogene 18:6910–6924.

Baust H, Schoke A, Brey A, Gern U, Los M, Schmid RM, Rottinger EM, Seufferlein T. 2003. Evidence for radiosensitizing by gliotoxin in HL-60 cells: Implications for a role of NF-kappaB independent mechanisms. Oncogene 22(54):8786–8796.

Brown GC, Borutaite V. 1999. Nitric oxide, cytochrome c and mitochondria. Biochemistry Society Symposium 66: 17–25.

Cai L, Klein JB, Kang YJ. 2000. Metallothionein inhibits peroxynitrite-induced DNA and lipoprotein damage. J Biol Chem 275:38957–38960.

Dubner D, Gisone P, Jaitavich I, Perez M. 1995. Free radicals production and estimation of oxidative stress related to gamma irradiation. Biol Trace Elem Res 47: 265–270.

Elsharkawy AM, Wright MC, Hay RT, Arthur MJ, Hughes T, Bahr MJ, Degitz K, Mann DA. 1999. Persistent activation of nuclear factor-kappaB in cultured rat hepatic stellate cells involves the induction of potentially novel Rel-like factors and prolonged changes in the expression of IkappaB family proteins. Hepatology 30(3): 761–769.

Fornace AJ Jr, Jackman J, Hollander MC, Hoffman-Liebermann DA, Liebermann B, 1992. Genotoxic-stress-response genes and growth-arrest genes. gadd, MyD, and other genes induced by treatments eliciting growth arrest. Ann NY Acad Sci 663:139–153.

Guillouf C, Grana X, Selvakumaran M, Hoffman B, Giordano A, Liebermann DA. 1995. Dissection of the genetic programs of p53-mediated G1 growth arrest and apoptosis: Blocking p53-induced apoptosis unmasks G1 arrest. Blood 85:2691–2698.

Gupta M, Gupta SK, Balliet AG, Hollander MC, Fornace AJ, Hoffman B, Liebermann DA. 2005. Hematopoietic cells from Gadd45a- and Gadd45b-deficient mice are sensitized to genotoxic-stress-induced apoptosis. Oncogene 24(48):7170–7179.

Gupta M, Gupta SK, Hoffman B, Liebermann DA. 2006. Gadd45a and Gadd45b protect hematopoietic cells from UV-induced apoptosis via distinct signaling pathways, including p38 activation and JNK inhibition. J Biol Chem 281(26):17552–17558.

Hanazaki K, Kajikawa S, Shimozawa N, Mihara M, Shimada K, Hiraguri M, Koide N, Adachi W, Amano J. 2000. Survival and recurrence after hepatic resection of 386 consecutive patients with hepatocellular carcinoma. J Am Coll Surg 191:381–388.

Holcik M, Yeh C, Korneluk RG, Chow T. 2000. Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. Oncogene 19:4174–4177.

Karin M, Lin A. 2002. NF-kappaB at the crossroads of life and death. Nat Immunol 3:221–227.

Kastan MB, Zhan Q, El-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, Fornace AJ Jr. 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 71:587–597.

Kim YM, de Vera ME, Watkins SC, Billiar TR. 1997. Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor-alpha-induced apoptosis by inducing heat shock protein 70 expression. J Biol Chem 272:1402–1411.

Kim YM, Chung HT, Simmons RL, Billiar TR. 2000. Cellular non-heme iron content is a determinant of nitric oxide-mediated apoptosis, necrosis, and caspase inhibition. J Biol Chem 275:10954–10961.

Kolb JP. 2000. Mechanisms involved in the pro- and anti-apoptotic role of NO in human leukemia. Leukemia 14:1685–1694.

Kroll M, Arenzana-Seisdedos F, Bachelerie F, Thomas D, Friguet B, Conconi M. 1999. The secondary fungal metabolite gliotoxin targets proteolytic activities of the proteasome. Chem Biol 6(10):689–698.

Kumar A, Middleton A, Chambers TC, Mehta KD. 1998. Differential roles of extracellular signal-regulated kinase-1/2 and p38 (MAPK) in interleukin-1β-and tumor necrosis factor-α-induced low density lipoprotein receptor expression in HepG2 cells. J Biol Chem 273:15742–15748.

Kwak JY, Han MK, Choi KS, Park IH, Park SY, Sohn MH, Kim UH, McGregor JR, Samlowski WE, Yim CY. 2000. Cytokines secreted by lymphokine-activated killer cells induce endogenous nitric oxide synthesis and apoptosis in DLD-1 colon cancer cells. Cell Immunol 203(2):84–94.

Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC. 1998. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 396:580–584.

Lin S, Fujii M, Hou DX. 2003. Rhein induces apoptosis in HL-60 cells via reactive oxygen species-independent mitochondrial death pathway. Arch Biochem Biophys 418:99–107.

Messmer UK, Reimer DM, Reed JC, Brune B. 1996. Nitric oxide induced poly (ADP-ribose) polymerase cleavage in RAW 264.7 macrophage apoptosis is blocked by Bcl-2. FEBS Lett 384:162–166.

Pahl HL, Krauss B, Schulze-Osthoff K, Decker T, Traenckner EB, Vogt M, Myers C, Parks T, Warring P, Muhlbacher A, Czernilofsky AP, Baeuerle PA. 1996. The immunosuppressive fungal metabolite gliotoxin specifically inhibits transcription factor NF-kappa. J Exp Med 183(4):1829–1840.

Papa S, Zazzeroni F, Pham CG, Bubici C, Franzoso G. 2004. Linking JNK signaling to NF-κB: A key to survival. J Cell Sci 117:5197–5208.

Richard JL, Debey MC, Chermette R, Pier AC, Hasegawa A, Lund A, Bratberg AM, Padhye AA, Connole MD. 1994. Advances in veterinary mycology. J Med Vet Mycol 32: 169–187.

Scherle PA, Jones EA, Favata MF, Daulerio AJ, Covington MB, Nurnberg SA, Magolda RL, Trzaskos JM. 1998. Inhibition of MAP Kinase Kinase prevents cytokine and prostaglandin E_2 production in lipopolysaccharide-stimulated monocytes. J Immunol 161:5681–5686.

Schmeck B, Zahlten J, Moog K, van Lak V, Huber S, Hocke AC, Opitz B, Hoffmann E, Kracht M, Zerrahn J, Hammerschmidt S, Rosseau S, Suttor PN, Hippenstiel S. 2004. Streptococcus pneumoniae-induced p38 MAPK-dependent phosphorylation of RelA at the interleukin-8 promoter. J Biol Chem 279:53241–53247.

Selvakumaran M, Lin HK, Miyashita T, Wang HG, Krajewski S, Reed JC, Hoffman B, Liebermann D. 1994. Immediate early up-regulation of bax expression by p53 but not TGF beta 1: A paradigm for distinct apoptotic pathways. Oncogene 9:1791–1798.

Shao R, Karunagaran D, Zhou BP, Li K, Lo SS, Deng J, Chiao P, Hung MC. 1997. Inhibition of nuclear factor-kappaB activity is involved in E1A-mediated sensitization of radiation-induced apoptosis. J Biol Chem 272(52): 32739–32742.

Steel GG, Peckham MJ. 1979. Exploitable mechanisms in combined radiotherapy-chemotherapy: The concept of additivity. Int J Radiat Oncol Biol Phys 5(1):85–91.

Sutton P, Newcombe NR, Waring P, Müllbacher A. 1994. In vivo immunosuppressive activity of gliotoxin, a metabolite produced by human pathogenic fungi. Infect Immun 62:1192–1198.

Szotowski B, Antoniak S, Goldin-Lang P, Tran QV, Pels K, Rosenthal P, Bogdanov VY, Borchert HH, Schultheiss HP, Rauch U. 2007. Antioxidative treatment inhibits the release of thrombogenic tissue factor from irradiation- and cytokine-induced endothelial cells. Cardiovasc Res 73(4):806–812.

Takayama T, Sekine T, Makuuchi M, Yamasaki S, Kosuge T, Yamamoto J, Shimada K, Sakamoto M, Hirohashi S, Ohashi Y, Kakizoe T. 2000. Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: A randomised trial. Lancet 356(9232):784–785.

Tamatani M, Ogawa S, Niitsu Y, Tohyama M. 1998. Involvement of Bcl-2 family and caspase-3-like protease in NO-mediated neuronal apoptosis. J Neurochem 71: 1588–1596.

Tamir S, deRojas-Walker T, Wishnok JS, Tannenbaum SR. 1996. DNA damage and genotoxicity by nitric oxide. Methods Enzymol 269:230–243.

Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS. 1998. NF-kappaB antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 281(5383):1680–1683.

Waring P, Beaver J. 1996. Gliotoxin and related epipolythiodioxopiperazines. Gen Pharmacol 27:1311–1316.

Waring P, Eichner RD, Müllbacher A, Sjaarda A. 1998. Gliotoxin induces apoptosis in macrophages unrelated to its antiphagocytic properties. J Biol Chem 263:18493–18499.

Yoo J, Ghiassi M, Jirmanova L, Balliet AG, Hoffman B, Fornace AJ Jr, Liebermann DA, Bottinger EP, Roberts AB. 2003. Transforming growth factor-β-induced apoptosis is mediated by Smad-dependent expression of GADD45b through p38 activation. J Biol Chem 278: 43001–43007.

Zhan Q, Lord KA, Alamo I Jr, Hollander MC, Carrier F, Ron D, Kohn KW, Hoffman B, Liebermann DA, Fornace AJ Jr. 1994. The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. Mol Cell Biol 14(4):2361–2371.