Targeting the Glyoxalase Pathway Enhances TRAIL Efficacy in Cancer Cells by Downregulating the Expression of Antiapoptotic Molecules

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Abstract
Methylglyoxal is an essential component in glycolysis and is known to be an inducer of apoptosis. Glyoxalase I (GLO1) metabolizes and inactivates methylglyoxal. GLO1 is known to be overexpressed in cancer cells and causes resistance to anticancer agents. We show for the first time that methylglyoxal treatment or the silencing of GLO1 enhances sensitivity to the promising anticancer agent TRAIL in malignant tumor cells. Methylglyoxal suppressed the expression of antiapoptotic factors, X-linked inhibitor of apoptosis protein (XIAP), survivin, cIAP1, Bcl-2, and Bcl-xL, without affecting TRAIL receptors, DR4 and DR5. Knockdown of XIAP or survivin by siRNA also enhanced TRAIL-induced apoptosis, indicating that downregulation of XIAP and survivin expression by methylglyoxal contributes to the enhancement of TRAIL activity. Furthermore, methylglyoxal decreased NF-κB activity with or without TRAIL treatment. On the other hand, the knockdown of GLO1 by siRNA enhanced TRAIL-induced apoptosis via the downregulation of XIAP and survivin expression. In conclusion, our results strongly suggest that sensitivity to TRAIL is increased by inhibition of the glyoxalase pathway and that the combination of TRAIL with methylglyoxal or glyoxalase inhibitors may be useful for a novel combination chemotherapy.

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Introduction
Glyoxalase I (GLO1) is known to metabolize and inactivate methylglyoxal, one of the side products of glycolysis (1, 2). The pathway is ubiquitous in all mammalian cells, and is involved in tissue maturation and cell death. Overexpression of GLO1 is observed in several types of malignant tumors, such as colon, breast, ovarian, and prostate cancer (3–8). GLO1 was identified as the most frequently amplified gene in 8.4% of human cancers (9). In addition, the multidrug resistance is conferred by the overexpression of GLO1 (9). GLO1 is particularly overexpressed in the more aggressive ovarian cancer or leukemia cells resistant to apoptosis caused by antitumor agents (9–11). Such findings suggest that GLO1 inhibitors may be potent chemotherapeutic agents causing an accumulation of methylglyoxal in cancer cells (9–12). Methylglyoxal is known to increase apoptosis (13–15).

TRAIL is a cytokine that plays an important role in immune surveillance for cancer (16, 17). TRAIL interacts with DR4 or DR5, leading to the activation of initiator caspase-8 or -10, which can directly evoke the cleavage of downstream effector caspases (16, 17). Bid, a proapoptotic Bcl-2 family member, is also cleaved by caspase-8 or -10 and then activates the mitochondrial apoptotic signaling pathway. Interestingly, TRAIL selectively induces apoptosis in various cancer cells in vitro and in vivo, with little or no toxicity in normal cells (18–20). Furthermore, a deficiency of TRAIL was found to accelerate the growth of malignant tumors in mice (21). Therefore, TRAIL is one of the most promising agents for cancer therapeutics, and clinical trials with recombinant human TRAIL and agonistic death receptor antibodies are ongoing (22). However, considerable numbers of cancer cells are resistant to apoptosis induced by TRAIL (23) because of the overexpression of antiapoptotic proteins, such as FLICE inhibitory protein, inhibitor of apoptosis proteins, and antiapoptotic Bcl-2 family proteins. Therefore, it is important to overcome this resistance in order to expand the therapeutic potential of TRAIL.

We here show, for the first time, that methylglyoxal or GLO1 inhibition overcomes resistance to TRAIL in cancer cells through downregulation of antiapoptotic proteins by suppression of nuclear factor-κB (NF-κB).
Materials and Methods

Reagents

Methylglyoxal was purchased from Sigma-Aldrich. Recombinant human TRAIL/Apo2L (amino acids 114–281) was obtained from PeproTech. zVAD-fmk, a pan-caspase inhibitor, was purchased from R&D Systems.

Cell culture

Human colon cancer SW480, HCT116, and lung cancer A549 cell lines were purchased from American Type Culture Collection. Cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS, 4 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C, with humidity and 5% CO2. Cell line authentication was not carried out by the authors within the last 6 months.

Plasmid preparation and luciferase assay

Luciferase constructs of the survivin promoter (pLuc-Surv/-3000) and its empty vector (pLuc) were a gift from Dr. Dario Altieli (University of Massachusetts Medical School, Worcester, MA). The X-linked inhibitor of apoptosis protein (XIAP) promoter construct and its empty vector (pGL2) were kindly provided by Dr. Taeg Kyu Kwon (Keimyung University, Taegu, Korea). Plasmid transfection and the luciferase assay were conducted as described previously (24). Briefly, the reporter plasmid or an empty vector plasmid (1 μg) was transfected into cells using the DEAE-dextran method (GE Healthcare). The blot of β-actin is a loading control. Arrows indicate cleaved protein bands. CT, control; TR, TRAIL. Data represent means ± SD of 3 determinations. *, P < 0.01.

Figure 1. Methylglyoxal (MG) sensitizes SW480 colon cancer cells to TRAIL-induced apoptosis. A, SW480 cells were treated with indicated concentrations of MG with or without 20 ng/mL TRAIL for 24 hours. Hypodiploid cells as shown in the sub-G1 population were analyzed by fluorescence-activated cell sorting. B, combined effects of 400 μmol/L MG and/or 20 ng/mL TRAIL for the period indicated were analyzed as in A. C, SW480 cells were treated with 400 μmol/L MG and/or 20 ng/mL TRAIL or 20 μmol/L zVAD-fmk for 24 hours. D, Western blotting of caspase-3 and PARP. Lysate from cells treated with 400 μmol/L MG and/or 20 ng/mL TRAIL or 20 μmol/L zVAD-fmk was used for Western blotting for caspase-3 or PARP. The blot of β-actin is a loading control.
Quantitative real-time reverse transcriptase (RT)-PCR was carried out using an RT-PCR system GeneAmp7300 (Applied Biosystems). TaqMan probe sets for XIAP, surviving, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were purchased from Applied Biosystems. The expression level of XIAP or survivin mRNA was normalized against the level of GAPDH mRNA in the same sample.

Detection of apoptosis
DNA fragmentation was quantified on the basis of the percentage of hypodiploid DNA (sub-G1). In brief, cells were pretreated with the indicated concentrations of methylglyoxal and/or TRAIL for 24 hours. After being washed with PBS, the collected cells were suspended in a 0.1% Triton-X 100/PBS solution. They were then treated with RNase A (Sigma) and the nuclei were stained with propidium iodide (Sigma). The DNA content was measured using FACSCalibur (Becton Dickinson). For each experiment, 10,000 events were analyzed. Cell Quest software (Becton Dickinson) was used to analyze the data.

Western blot analysis
Whole cell lysate containing 50 µg of protein was separated on a 7.5% to 12.5% SDS-PAGE for electrophoresis, and blotted onto polyvinylidene difluoride membranes (Millipore). The following antibodies were purchased from the indicated sources: rabbit polyclonal antibodies for DR5 and DR4 (Prosci), caspase-3 (Cell Signaling Technology), Bcl-XL, cellular inhibitor of apoptosis protein (cIAP1), Sp1 and GLO1 (Santa Cruz Biotechnology), survivin (R&D systems), NF-κB p65 (Merck/Calbiochem), and mouse monoclonal antibodies for XIAP; R&D Systems), PARP, Bcl-2 (Santa Cruz), and β-actin (Sigma). The signal was detected with an ECL Western blot analysis system (GE Healthcare).

Preparation of nuclear extracts
Cells were washed with ice-cold PBS and resuspended in buffer A [10 mmol/L HEPES (pH 7.8), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1% NP-40, 1 mmol/L dithiothreitol (DTT), 0.5 mmol/L phenylmethylsulfonylfluoride (PMSF), 2 µg/mL apro tin, and 2 µg/mL leupeptin]. After centrifugation at 5,000 × g for 1 minute, the nuclear pellets were resuspended in buffer C [50 mmol/L HEPES (pH 7.8), 420 mmol/L KCl, 0.1 mmol/L EDTA, 5 mmol/L methylglyoxal, 20% glycerol, 1 mmol/L DTT, 0.5 mmol/L PMSF, 2 µg/mL apro tin, and 2 µg/mL leupeptin] and incubated for 30 minutes at 4°C. The nuclear debris was spun down at 15,000 × g for 15 minutes, and the supernatant was stored in aliquots at −80°C. Protein concentrations of nuclear extracts were determined using the Protein Assay Kit (Bio-Rad Laboratories).

siRNAs
The XIAP, surviving, and LacZ siRNA were described previously (25, 26) and synthesized by Proligo. GLO1

siRNA was purchased from Santa Cruz Biotechnology. The cells were seeded into the medium at a density of 50% to 60%. The siRNA was transfected with HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s protocol.

Measurement of the NF-κB (p65) DNA–binding activity
For the measurement of the NF-κB (p65) DNA–binding activity, we used PahtDetect cis-reporting plasmids pNFκBluci (Stratagene), which contained the luciferase reporter gene driven by the basic promoter element TATA box with 5 repeats of the consensus binding sequence for the NF-κB, according to the manufacturer’s instructions.

Statistical analysis
The measurements were repeated 3 times and statistically analyzed using a 2-tailed, paired Student t test. Samples were considered significantly different when P < 0.05.

Results
Methylglyoxal enhances TRAIL-induced apoptosis in SW480 colon cancer cells
At first, we investigated the effect of methylglyoxal on TRAIL-induced apoptosis by measuring the sub-G1 population reflecting hypodiploid cells. Methylglyoxal or exogenous recombinant human TRAIL alone slightly
induced apoptosis in SW480 colon cancer cells. However, the combined treatment with TRAIL and methylglyoxal markedly induced apoptosis in a dose- and time–dependent manner (Fig. 1A and B), indicating that methylglyoxal sensitizes SW480 cells to TRAIL-induced cell death.

The sub-G₁ population induced by methylglyoxal and TRAIL reflects caspase-dependent apoptosis

As shown in Fig. 1C, the pan-caspase inhibitor zVAD-fmk attenuated an increase in the sub-G₁ population induced by the combination of TRAIL and methylglyoxal, indicating that the sub-G₁ population reflects caspase-dependent apoptosis. We also showed that the combination of methylglyoxal and TRAIL cleaved caspase-3 and a caspase substrate PARP, whereas zVAD-fmk blocked these cleavages (Fig. 1D). These results indicate that methylglyoxal synergistically enhanced TRAIL-induced apoptosis in a caspase-dependent manner.

Methylglyoxal downregulates the expression of antiapoptotic proteins in SW480 cells

To elucidate the underlying mechanisms by which methylglyoxal enhanced TRAIL-induced apoptosis, we examined the changes of protein expression by methylglyoxal in SW480 cells. First, we carried out Western blotting for several antiapoptotic proteins. Methylglyoxal considerably inhibited the expression of XIAP, cIAP1, survivin, Bcl-2, and Bcl-X₁ in a dose- and time–dependent manner (Fig. 2A and B), whereas cIAP2, Bax, and Bak were not significantly changed (data not shown). Next, we examined expression levels of TRAIL receptors, DR4 and DR5. As shown in Fig. 2C, methylglyoxal did not apparently induce the expression of DR4 and DR5, whereas methylglyoxal at 400 μmol/L slightly induced DR5 expression. Nevertheless, methylglyoxal at 400 μmol/L did not change the cell surface expression of DR4 and DR5 measured by immunostaining and flow cytometry (Supplementary Fig. S1). Therefore, the expression levels of DR4 and DR5 were not correlated with apoptotic levels shown in Fig. 1A. These results suggest that the sensitization of TRAIL-induced apoptosis by methylglyoxal is relevant to the inhibition of antiapoptotic proteins.

Methylglyoxal suppresses the activation of NF-κB in SW480 cells

Moreover, downregulation of XIAP or survivin expression using siRNA increased the apoptosis by TRAIL in SW480 cells (Fig. 3A), indicating that methylglyoxal induced apoptosis in SW480 colon cancer cells. However, the combined treatment with TRAIL and methylglyoxal markedly induced apoptosis in a dose- and time–dependent manner (Fig. 1A and B), indicating that methylglyoxal sensitizes SW480 cells to TRAIL-induced cell death.

**Figure 3.** Methylglyoxal (MG) downregulates XIAP and survivin expression via suppression of NF-κB activity. A, twenty-four hours after the transfection, efficiency of XIAP and survivin siRNA was confirmed by Western blotting (right). Twenty-four hours after the knockdown of XIAP or survivin, SW480 cells were treated with 10 ng/mL TRAIL for 24 hours, and the sub-G₁ population was analyzed by fluorescence-activated cell sorting. B, cells were treated with MG for 24 hours, and XIAP and survivin mRNA were analyzed by real-time RT-PCR. C, luciferase reporter plasmids containing the XIAP or survivin promoter were transfected into SW480 cells. After the MG treatment, a luciferase assay was carried out. D and E, SW480 cells were treated with 20 ng/mL TRAIL and/or 400 μmol/L MG and nuclear extracts were prepared. NF-κB DNA–binding activity in nuclear extracts was assayed as described in Materials and Methods D, using nuclear extracts, Western blotting for the NF-κB subunit p65 was conducted (E), Sp1 is a loading control. Data represent means ± SD of 3 determinations. *, P < 0.01

**Figure 4.** Knockdown of GLO1 sensitizes SW480 cells to TRAIL-induced apoptosis. A, GLO1 siRNA (20 nmol/L) was transfected with HiPerFect reagent. Twenty-four hours after the transfection, cells were treated with 10 ng/mL TRAIL for 24 hours and the sub-G₁ population was analyzed by fluorescence-activated cell sorting. Data represent means ± SD of 3 determinations. *, P < 0.01. B, Western blotting for GLO1, XIAP, and survivin was carried out 24 hours after the transfection. TR, TRAIL.
sensitizes TRAIL-induced apoptosis at least partially through the inhibition of XIAP and survivin. We next examined how methylglyoxal decreased the levels of XIAP and survivin. Methylglyoxal substantially down-regulated XIAP and survivin mRNA expression in a dose-dependent manner (Fig. 3B). Furthermore, the promoter activities of XIAP and survivin were decreased by methylglyoxal in SW480 cells (Fig. 3C), suggesting that...
methylglyoxal transcriptionally downregulated the expression of XIAP and survivin. We next investigated NF-xB activity on the basis of the affinity of NF-xB for DNA in nuclear extracts following methylglyoxal treatment. As shown in Fig. 3D, methylglyoxal suppressed the NF-xB activity. Although TRAIL slightly increased NF-xB activity, methylglyoxal was able to attenuate the NF-xB activity even in combination with TRAIL. Consistent with the result in Fig. 3D, the amount of a NF-xB subunit, p65, in nuclei was decreased by methylglyoxal treatment with or without TRAIL (Fig. 3E), suggesting that downregulation of p65 expression by methylglyoxal causes the inactivation of NF-xB.

**Knockdown of GLO1 sensitizes SW480 cells to TRAIL-induced apoptosis**

We next examined whether knockdown of GLO1 by siRNA sensitized SW480 cells to TRAIL-induced apoptosis, and found that GLO1 siRNA significantly enhanced the apoptosis induced by TRAIL (Fig. 4A). In addition, GLO1 siRNA efficiently decreased the expression of XIAP and survivin (Fig. 4B). These results indicate that knockdown of GLO1 enhances TRAIL-induced apoptosis through downregulation of XIAP and survivin expression.

**Methylglyoxal or knockdown of GLO1 enhances TRAIL sensitivity in colon cancer HCT116 and lung cancer A549 cells**

We showed that methylglyoxal or knockdown of GLO1 sensitized colon cancer SW480 cells to TRAIL-induced apoptosis. We then used 2 other human cancer cell lines, colon cancer HCT116 and lung cancer A549. Methylglyoxal enhanced TRAIL-induced apoptosis and downregulated the expression of XIAP and survivin in both HCT116 and A549 cells (Fig. 5A and B). Furthermore, methylglyoxal with or without TRAIL diminished NF-xB activity in both cell lines (Fig. 5C). GLO1 siRNA also sensitized HCT116 and A549 cells to TRAIL-induced apoptosis (Fig. 5D), and decreased levels of XIAP and survivin (Fig. 5E). These results suggest that our finding is not specific to only 1 cell line.

**Discussion**

TRAIL is one of the most attractive candidates for cancer treatment and phase I/II studies are ongoing (22). However, considerable numbers of malignant tumor cell lines are resistant to apoptosis induced by TRAIL (23). It is therefore important to find agents to overcome the resistance to TRAIL. In the present study, we first found that GLO1 inhibition and MG are effective in overcoming TRAIL resistance (Figs. 1, 4, and 5F). Furthermore, we showed that GLO1 inhibition and MG decreased NF-xB activity with or without TRAIL treatment. NF-xB is also known to upregulate the expression of several antiapoptotic proteins, such as IAP family proteins (27-30) or Bcl family proteins (31). Taken together, it was suggested that GLO1 inhibition or methylglyoxal may cause apoptosis through downregulation of NF-xB activity. We additionally found that methylglyoxal enhanced sensitivity to TRAIL through the suppression of NF-xB activity and expression of antiapoptotic proteins (Figs. 3, 4, and 5F). Because methylglyoxal was known to increase reactive oxygen species (ROS; ref. 15), we examined whether methylglyoxal induced production of ROS and the ROS production by methylglyoxal was related to the enhancement of TRAIL-induced apoptosis. As shown in Supplementary Fig. S2, there was no correlation between ROS production and the sensitization of TRAIL-induced apoptosis by methylglyoxal.

We have previously reported that combined agents on the basis of molecular mechanisms are effective, and proposed a new concept termed "combination-oriented molecular-targeting prevention and therapy of cancer" (32). We also found that many agents upregulate DR5 expression and overcome TRAIL resistance in cancer cells (24, 33, 34). With this concept, it is effective for enhancement of TRAIL efficacy not only to upregulate the expression of TRAIL receptors but also to inhibit antiapoptotic gene expression related to the signaling pathway for TRAIL. We here showed that inhibition of antiapoptotic genes, XIAP and survivin, by GLO1 inhibition or methylglyoxal overcame resistance to TRAIL in cancer cells.

In summary, we showed that GLO1 inhibition or methylglyoxal enhances TRAIL-induced apoptosis via downregulation of antiapoptotic gene expression mediated by NF-xB inhibition. We therefore propose a novel clinical strategy for overcoming TRAIL resistance using GLO1 inhibitors, which should be developed by pharmaceutical companies.
References


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