Oxaliplatin enhances TRAIL-induced apoptosis in gastric cancer cells by CBL-regulated death receptor redistribution in lipid rafts

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1. Introduction

Although chemotherapy improves the survival of advanced gastric cancer patients, the median overall survival is no more than 12 months [1,2]. Accordingly, new therapeutic methods are worthy of development. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) family, and induces apoptosis in many cancer cells but spares most normal cells. However, gastric cancer cells are insensitive to TRAIL [3,4]. TRAIL binding to receptor 4 (DR4) and death receptor 5 (DR5) results in receptor aggregation, and the death-inducing signaling complex (DISC) formation, followed by sequential apoptosis [5,6]. Recent studies have shown that lipid rafts provide a membrane platform for death receptors aggregation and play an important role in initiating death signaling transmission [7,8]. This implies that the dysfunction of lipid raft maybe is one reason that gastric cancer cells are insensitive to TRAIL.

Some chemotherapeutic drugs have been shown to sensitize cancer cells to TRAIL [9–11]. Oxaliplatin is a third-generation platinum-containing drug, and oxaliplatin-based regimens have significantly improved efficacy compared with cisplatin-based regimens in the treatment of colorectal cancer. Oxaliplatin is also regarded as a promising drug for the treatment of gastric cancer [12,13]. Cisplatin has the ability to sensitize colon cancer cells to CD95 ligand by triggering lipid raft aggregation [14]. Whether oxaliplatin can enhance the sensitivity of gastric cancer cells to TRAIL by clustering death receptor into lipid rafts is unknown.

The casitas B-lineage lymphoma (Cbl) family of ubiquitin ligases is an important regulator of lipid rafts. It has been demonstrated that c-Cbl and Cbl-b can sequester signaling molecules from lipid rafts, which results in ineffective lipid raft aggregation in T cells and mast cells [15–17]. Moreover, the loss of Cbl-b in T cells triggers receptor clustering and lipid raft aggregation [18]. However, whether c-Cbl and Cbl-b can negatively regulate lipid raft aggregation and therefore influence the sensitivity of gastric cancer cells to TRAIL is not yet clear.
To sensitize gastric cancer cells to TRAIL, we treated them with a combination of oxaliplatin and TRAIL. Our results for the first time showed that oxaliplatin enhanced TRAIL-induced gastric cancer cells apoptosis at least partially through Cbl-regulated death receptor redistribution in lipid rafts.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant human TRAIL was purchased from Cytolab/Peprotech Asia (USA). Oxaliplatin was obtained from Sanofi-Aventis. The FITC-conjugated cholera toxin B subunit was from Sigma Chemical Co. Rhodamine-conjugated goat anti-mouse IgG, anti-DR4, anti-DR5, anti-procaspase-3, anti-procaspase-8, anti-Bax, anti-Bcl-2, and anti-Cbl-b antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-c-Cbl antibody was purchased from Transduction Laboratories (Lexington, KY).

2.2. Cell cultures and transfection

The gastric cancer cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 medium (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37 °C under an atmosphere of 95% air and 5% CO2. For transfection, cells were seeded into six-well plates (5 × 10^4 cells/well) without antibiotics. After 24 h, cells were transfected with various plasmids using Lipofectamine 2000 reagent (Invitrogen, USA), according to the manufacturer’s instructions.

2.3. MTT assay

The cells were seeded at 5 × 10^4 cells/well in 96-well plates and then exposed to oxaliplatin and/or TRAIL. Thereafter, 25 μL of MTT solution (5 mg/mL) was added to each well and the cells were incubated for 4 h. The percentage of apoptotic cells was quantitated by flow cytometry. (A) Cell viability was determined by MTT assay. (B) The percentage of apoptotic cells was quantitated by flow cytometry. (C) The mitochondrial membrane potential was analyzed by flow cytometry. (D) The expression of procaspase-3, procaspase-8, Bax and Bcl-2 proteins was analyzed by western blotting.

Fig. 1. Oxaliplatin and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) synergistically affected cell viability, cell apoptosis, and mitochondrial membrane potential in gastric cancer cells. TRAIL was regarded as a positive control. MGC803, BGC823, and SGC7901 cells were treated with oxaliplatin (at the respective IC50 doses) and TRAIL (100 ng/mL) for 24 h. MGC803 cells were treated with 5-FU (2 μg/mL) and TRAIL (100 ng/mL) for 48 h. (A) Cell viability was determined by MTT assay. (B) The percentage of apoptotic cells was quantitated by flow cytometry. (C) The mitochondrial membrane potential was analyzed by flow cytometry. (D) The expression of procaspase-3, procaspase-8, Bax, and Bcl-2 was analyzed by western blotting.
incubated for another 4 h at 37 °C. Then, the cells were lysed in 200 μL of dimethylsulfoxide (DMSO) and the optical density (OD) was measured at 570 nm with a microplate reader (Model 550, Bio-Rad Laboratories, USA).

2.4. Flow cytometry analysis

The cells were seeded at 3 × 10⁵ cells/well in six-well plates and then exposed to oxaliplatin and/or TRAIL. The cells were collected and incubated with 5 μL Annexin V and 10 μL PI for 15 min in the dark. Moreover, mitochondrial depolarization was determined by means of the cationic lipophilic fluorochrome DiOC6. Cells were collected and incubated with 20 nm DiOC6 for 15 min in the dark. Finally, the samples were evaluated by flow cytometry.

2.5. Western blot analysis

Cells were solubilized in 1% Triton lysis buffer on ice. Cell lysate proteins were separated by polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membrane (Immoblin-P, Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk in TBST buffer, incubated with the indicated antibodies and reacted with horseradish-peroxidase-conjugated secondary antibodies. The immunoreactive proteins were visualized with chemiluminescence reagent (SuperSignal Western Pico Chemiluminescent Substrate, Pierce, USA).

2.6. Immunofluorescence microscopy

The cells were seeded and treated in Lab-Tek chamber slides (Nunc S/A, Polylabo, Strasbourg, France) and then fixed in 3.3% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 3 min and blocked with 5% bovine serum albumin (BSA). For double staining, the cells were primed with anti-cholera toxin B subunit, anti-DR4, or anti-DR5 antibody for 1 h and then incubated with rhodamine-conjugated goat anti-mouse IgG for 45 min. The cells were mounted using the SlowFade Antifade Kit (Molecular Probes, Eugene, OR), and analyzed by confocal fluorescence microscopy (PV1000S-SIM/IX81, Olympus, Japan).

3. Results

3.1. Oxaliplatin enhanced TRAIL-induced apoptosis of MGC803, BGC823, and SGC7901 cells

The synergistic activity of 5-FU + TRAIL on gastric cancer cells has been demonstrated [19], so 5-FU was regarded as a positive control. Our results showed that 100 ng/mL TRAIL resulted in a slight reduction in cell viability and no more than 6% cell apoptosis in three cell lines. Treatment with oxaliplatin (at the respective IC₅₀ doses) plus TRAIL (100 ng/mL) for 24 h resulted in a significant reduction in cell viability and increase in cell apoptosis compared with treatment with oxaliplatin or TRAIL alone (Fig. 1A and B). When two drugs were used in combination after 16 h, a higher degree of mitochondrial depolarization, cleavage of procaspase-3 and procaspase-8 and decreased expression of Bcl-2 were detected (Fig. 1C and D). These results indicated that oxaliplatin enhanced TRAIL-induced apoptosis through activation of mitochondria and executive caspases in gastric cancer cells.

3.2. Oxaliplatin promoted DR4 and DR5 clustering into aggregated lipid rafts

Recent study has shown that cisplatin induces CD95 clustering into lipid rafts [14]. This led us to investigate whether oxaliplatin can cluster death receptor into lipid rafts. Oxaliplatin (22.56 μg/mL) and TRAIL (100 ng/mL) were used in MGC803 cells for the following experiments. As shown in Fig. 2, exposure to TRAIL alone for 16 h did not induce obvious lipid raft aggregation or DR4 or DR5 clustering. The combined treatment with oxaliplatin and TRAIL showed similar results.
3.3. Nystatin partially prevented oxaliplatin-induced lipid raft aggregation and DR4 and DR5 clustering, and decreased the apoptosis

To investigate the effects of lipid raft aggregation on cell apoptosis, nystatin, a cholesterol-sequestering agent that disrupted lipid rafts, was used for next experiment. Interestingly, pretreatment for 1 h with 50 μg/mL nystatin partially prevented lipid raft aggregation and DR4 and DR5 clustering induced by oxaliplatin or oxaliplatin + TRAIL (Fig. 3A) and significantly suppressed oxaliplatin + TRAIL-induced apoptosis (Fig. 3B). These results suggested that oxaliplatin-induced DR4 and DR5 clustering in aggregated lipid rafts facilitated TRAIL-induced gastric cancer cell apoptosis.

3.4. Oxaliplatin downregulated the expression of c-Cbl and Cbl-b

Recent studies have shown that ubiquitin ligases c-Cbl and Cbl-b are negative regulators of lipid rafts [15,16]. Our data showed that oxaliplatin alone strongly reduced c-Cbl and Cbl-b protein expression.
levels after 16 and 24 h (Fig. 4A). Exposure to oxaliplatin + TRAIL induced the downregulation of c-Cbl and Cbl-b, similar to exposure to oxaliplatin alone at 16 h (Fig. 4B). These results suggested that oxaliplatin-induced DR4 and DR5 clustering into lipid rafts might be triggered by downregulation of c-Cbl and Cbl-b.

3.5. Overexpression of c-Cbl and Cbl-b partially reversed oxaliplatin-induced lipid raft aggregation

To confirm that c-Cbl and Cbl-b negatively regulate lipid raft aggregation, we transiently transfected c-Cbl and Cbl-b into MGC803 cells. As shown in Fig. 5A, lipid raft aggregation was partially reversed by c-Cbl, Cbl-b, and c-Cbl and Cbl-b compared with the control. The expression of c-Cbl and Cbl-b was shown in Fig. 5B. These results indicated that c-Cbl and Cbl-b partially reversed oxaliplatin-induced lipid raft aggregation.

4. Discussion

TRAIL selectively induces cancer cell apoptosis. Oxaliplatin has been shown to sensitize colon cancer cells to TRAIL [20,21]. In the present study, when MGC803, BGC823, and SGC7901 cells were treated with 100 ng/mL TRAIL for 24 h, the proportion of apoptotic cells was no more than 6%, indicating that three cell lines were insensitive to TRAIL. But oxaliplatin significantly enhanced TRAIL-induced gastric cancer cells apoptosis through intrinsic and extrinsic apoptosis pathways.

Lipid rafts provide a membrane platform for death receptor aggregation and trigger death signaling transmission. In our study, TRAIL failed to induce obvious lipid raft aggregation or DR4 or DR5 clustering. This was probably one reason that gastric cancer cells were insensitive to TRAIL. Some agents, such as cisplatin, edelfosine and quercetin, can cause death receptor clustering into lipid rafts [22,23]. Our results showed that oxaliplatin promoted DR4 and DR5 clustering into aggregated lipid rafts and enhanced TRAIL-induced apoptosis in gastric cancer cells. The cholesterol-sequestering agent nystatin partially prevented lipid raft aggregation and DR4 and DR5 clustering, and reduced the apoptosis. These data suggested that the integrity of lipid rafts was necessary for death receptor clustering.

Ubiquitin ligases c-Cbl and Cbl-b are negative regulators of lipid rafts. Our previous studies showed that translocation of Cbl-b into the lipid raft regulated the functions of some signaling molecules and resulted in inefficient lipid raft aggregation [16,17]. Krawczyk et al. confirmed that the loss of Cbl-b resulted in the enhancement of lipid raft aggregation [18]. In the present study, oxaliplatin significantly downregulated the expression of c-Cbl and Cbl-b. Meanwhile lipid raft aggregation occurred. Overexpression of c-Cbl and Cbl-b was analyzed by western blotting.
Cbl-b partially suppressed lipid raft aggregation. These results suggested that the translocation of Cbl proteins played a role in regulating lipid raft aggregation.

In summary, our results demonstrated that oxaliplatin enhanced TRAIL-induced apoptosis in gastric cancer cells. The synergistic antitumor mechanism was associated with oxaliplatin-induced lipid raft aggregation and DR4 and DR5 clustering. DR4 and DR5 redistributed in lipid rafts in response to oxaliplatin-induced downregulation of the Cbl family.

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References