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Cetuximab enhances TRAIL-induced gastric cancer cell apoptosis by promoting DISC formation in lipid rafts

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ABSTRACT

TRAIL is a member of the tumor necrosis factor family that selectively induces cancer cell apoptosis. However, gastric cancer cells are insensitive to TRAIL. Our and others studies showed that the inhibition of EGFR pathway activation could increase the sensitivity of TRAIL in cancer cells. But the detailed mechanism is not fully understood. In the present study, compared with TRAIL or cetuximab (an anti-EGFR monoclonal antibody) alone, treatment with the TRAIL/cetuximab combination significantly promoted death receptor 4 (DR4) clustering as well as the translocation of both DR4 and Fas-associated death domain-containing protein (FADD) into lipid rafts. This in turn resulted in caspase-8 cleavage and the formation of the death-inducing signaling complex (DISC) in these lipid rafts. Cholesterol-depletion with methyl-β-cyclodextrin partially prevented DR4 clustering and DISC formation, and thus partially reversed apoptosis induced by the TRAIL/cetuximab dual treatment. These results indicate that cetuximab increases TRAIL-induced gastric cancer cell apoptosis at least partially through the promotion of DISC formation in lipid rafts.

1. Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a highly selective molecule which induces apoptosis in malignant, but not in normal cells. Moreover, some phase II clinical studies have shown that an agonistic antibody specific for TRAIL receptors is safe and well-tolerated in patients with advanced colorectal cancer, non-small cell lung cancer and non-Hodgkin's lymphoma [1–3]. However, the sensitivities to TRAIL differ in various cancer cells. For example, while Jurkat T leukemia cells are very sensitive to TRAIL, most gastric cancer cells are relatively resistant [4]. Thus, inherent tumor resistance is a major barrier for effective TRAIL-targeted therapy. Accordingly, identification of the mechanisms underlying different TRAIL sensitivity in cancer could be the key to reversing resistance.

Stimulation of TRAIL results in death receptor 4 (DR4) and death receptor 5 (DR5) aggregation and recruitment of the adaptor molecule Fas-associated death domain-containing protein (FADD) through interaction between the death domains of FADD and TRAIL receptor. The death domain of FADD then binds to an analogous domain of caspase-8 to form the death-inducing signaling complex (DISC), which activates downstream caspases and leads to apoptosis [5]. DISC formation is the most upstream event of TRAIL-triggered apoptosis [6]. It has been reported that chemotherapeutic drugs, such as cisplatin, etoposide and 5-fluorouracil, overcome TRAIL resistance at the DISC level in cervical carcinoma cells [7]. Likewise, increased caspase-8 recruitment and activation of DISC have been associated with the restoration of TRAIL sensitivity in colon carcinoma cells [8]. Thus, effective DISC formation plays an important role in triggering the TRAIL signaling pathway.

Lipid rafts, which are enriched in cholesterol, sphingomyelins, and glycosphingolipids, provide a vital platform for the membrane trafficking and signal transduction required in many cellular processes [9,10]. Our previous study demonstrated that the dysfunction of lipid rafts inhibits TRAIL-induced death receptor clustering in gastric cancer cells [11]. As the common membrane platform, cross-talk between many membrane receptor molecules occurs in lipid rafts where they can influence the function of each other. For example, cross-talk between epidermal growth factor receptor (EGFR) and T-cadherin, a new membrane receptor for adiponectin, promotes T-cadherin localization to intercellular contacts [12]. EGFR is also localized in lipid rafts [13]. Our recent study showed that treatment of gastric cancer cells with TRAIL induces EGFR translocation into lipid rafts in accompaniment with EGFR activation. Moreover, the anti-EGFR antibody cetuximab was demonstrated to increase cell sensitivity to TRAIL by inactivation of the EGFR pathway [14]. In addition, Bremer et al. reported that antibody fragment binding of scFv425:sTRAIL to EGFR immobilized

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scFv425:sTRAIL on the cell surface of EGFR-positive tumor cells and converted scFv425:sTRAIL into a membrane bound form that can efficiently initiate apoptosis by cross-linking of the agonistic TRAIL receptors. Moreover, the synergistic induction of apoptosis by scFv425:sTRAIL and Iressa (an EGFR-specific tyrosine kinase inhibitor) was detected in cancer cells [15]. However, the detailed mechanism of regulation between the TRAIL receptor and EGFR is unclear. Also, whether the blockade of EGFR influences TRAIL receptor-mediated DISC formation in lipid rafts is as yet unknown.

In the present study, we demonstrated that cetuximab increases TRAIL-induced apoptosis at least partially through the promotion of DISC formation in lipid rafts in gastric cancer cells.

2. Materials and methods

2.1. Reagents and antibodies

Cetuximab was obtained from Merck KgaA (Germany). Recombinant human TRAIL/Apo2L was purchased from Cytolab/Peprotech Asia (USA). The fluorescein isothiocyanate (FITC)-conjugated cholera toxin B subunit from *Vibrio cholerae* was from Sigma Chemical Co (USA). Alexa Fluor 568-conjugated goat anti-mouse antibody was obtained from (Invitrogen, USA). Anti-poly (ADP-ribose) polymerase (PARP) and anti-caspase-3 antibodies were obtained from Cell Signaling Technology (USA). Other antibodies were all obtained from Santa Cruz Biotechnology (USA).

2.2. Cell cultures

The gastric cancer MGC803 and SGC7901 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) at 37 °C under an atmosphere of 95% air and 5% CO_2. The cells were routinely subcultured every 2 or 3 days.

2.3. Flow cytometry analysis

MGC803 and SGC7901 cells were seeded in six-well plates and exposed to cetuximab and TRAIL. The cells were collected and fixed with ice-cold 70% ethanol for 12 h, then incubated with 20 μg/mL RNase A at 37 °C for 30 min and 10 μg/mL propidium iodide (PI) for 30 min in the dark. Finally, the samples were evaluated by flow cytometry and the data were analyzed by WinMDI software.

2.4. Western blot analysis

MGC803 and SGC7901 cells were solubilized in 1% Triton lysis buffer on ice and quantified with Lowry method. Cell lysate proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membrane. The membranes were blocked with 5% skim milk in TBST buffer at room temperature for 2 h and incubated overnight at 4 °C with the indicated primary antibodies. Then the membranes were incubated with the appropriate secondary antibodies for 30 min at room temperature. The proteins were detected with chemiluminescence reagent and visualized with the Electrophoresis Gel Imaging Analysis System (DNR Bio-Imaging Systems, Israel).

2.5. Isolation of lipid rafts

The cells were solubilized in 150 μL of prechilled TXNE buffer containing protease inhibitors (chymostatin, leupeptin, antipain, and pepstatin) for 20 min on ice. Then, the cells were extracted and moved into 35% Optiprep (Axis-shield, Norway) in polyallomer ultra tubes by adding 210 μL of 60% Optiprep/0.1% Triton X-100. Finally, the cell lystaes were covered with 3.5 mL 30% Optiprep in TXNE buffer and 300 μL TXNE buffer. After spin (4 h, 200,000*g*, 4 °C) in the ultracentrifuge, six fractions were collected from the top. The proteins in fractions 1–2 were taken as the lipid raft fractions.

2.6. Immunofluorescence microscopy.

The cells were seeded and treated in Lab-Tek chamber slides and then fixed in 3.3% paraformaldehyde for 20 min, and blocked with 5% bovine serum albumin in PBS. For staining, the cells were primed with anti-cholera toxin B subunit and anti-DR4 mouse monoclonal antibody for 1 h and then incubated with Alexa Fluor 568-conjugated goat anti-mouse IgG for 45 min. Finally, the cells were mounted using the SlowFade Antifade Kit and analyzed by laser confocal scanning microscope (TCS SP2/AOBS, LEICA, Germany).

2.7. Statistical analysis

Data were confirmed in three independent experiments. SPSS18.0 computer software was used for statistical analysis. Differences between groups were compared using Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cetuximab enhances TRAIL-induced apoptosis by promotes DR4 clustering and DISC formation in lipid rafts of gastric cancer cells

TRAIL (100 ng/ml) and cetuximab (1 μg/ml) were used for treatment as per our previous study [14,16]. TRAIL alone mildly induced apoptosis, while cetuximab alone had no significant effect. Compared with TRAIL or cetuximab alone, treatment with TRAIL plus cetuximab significantly increased apoptosis in MGC803 and SGC7901 cells (respectively, $P < 0.05$). To clarify whether the efficacy of cetuximab on TRAIL-induced apoptosis is associated with DR4 clustering and DISC formation in lipid rafts, the distribution of death receptors in lipid rafts was observed by confocal scanning microscopy, and the constituents of DISC were isolated by ultracentrifugation. The locations of lipid rafts (fractions 1–2, Fig. 1B) were determined using caveolin-1. As shown in Fig. 1A, exposure to TRAIL alone for 16 h did not induce obvious lipid raft aggregation or DR4 clustering in MGC803 cells. However, cetuximab alone promoted the localization of DR4 in aggregated lipid rafts. The combined treatment with cetuximab and TRAIL showed further significant DR4 aggregation in lipid rafts. In addition, in comparison to untreated MGC803 cells, exposure to TRAIL alone induced translocation of DR4 as well as slight translocation of FADD into lipid raft fractions (1 and 2, Fig. 1B). At the same time, caspase-8 was slightly cleaved in the lipid raft fractions (Fig. 1B). Although cetuximab alone induced the translocation of DR4 and procaspase-8, as well as the slight translocation of FADD into the lipid raft fractions (1 and 2, Fig. 1B), cleavage of caspase-8 was not detected. Interestingly, treatment with cetuximab and TRAIL induced greater translocation of DR4 and FADD into the lipid raft fractions (1 and 2, Fig. 1B), and the cleavage of caspase-8 was also observed (Fig. 1B). Thus the data suggest that cetuximab combined with TRAIL induces DISC formation in lipid rafts, which facilitates TRAIL-triggered apoptosis in gastric cancer cells.

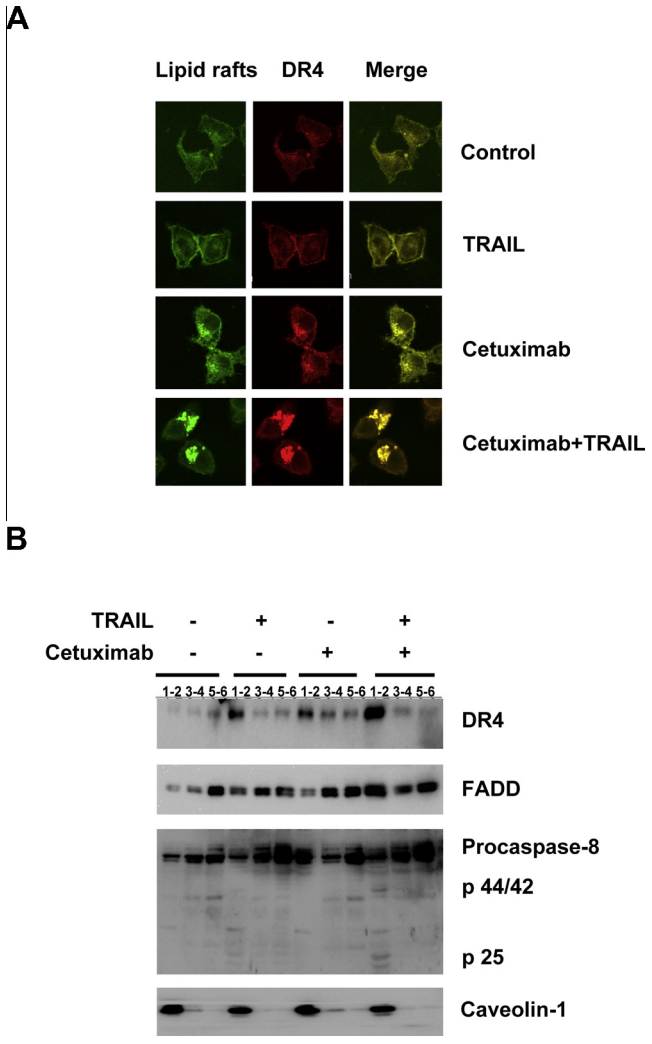

Fig. 1. Cetuximab promoted TRAIL-induced DR4 clustering and DISC formation in lipid rafts. (A) MGC803 cells were treated with 100 ng/ml TRAIL, 1 μg/mL cetuximab, or a combination of both for 16 h. Then, the cells were stained with anti-cholera toxin B subunit and anti-DR4 antibody for 1 h, and then incubated with Alexa Fluor 568-conjugated antibody for 45 min. Finally, the cells were analyzed by confocal fluorescence microscopy. Original magnification, ×63. (B) MGC803 cells were treated with under similar conditions to those in (A). The cells were then fractionated by the ultracentrifuge. Locations of lipid rafts (fractions 1–2) were determined using caveolin-1. The indicated proteins were analyzed by Western blot.

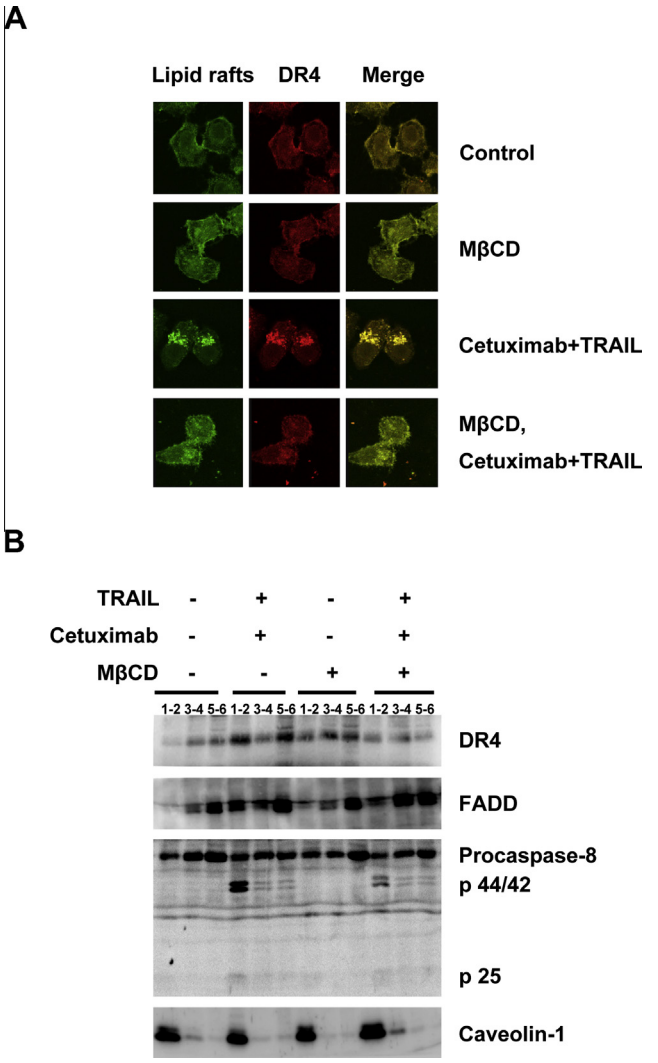

Fig. 2. MβCD partially prevented DR4 clustering and DISC formation in lipid rafts induced by cetuximab and TRAIL. (A) MGC803 cells were exposed to 2.5 mg/mL MβCD for 30 min in serum-free medium. The cells were then washed with PBS, resuspended in complete culture medium and treated with TRAIL (100 ng/mL) and cetuximab (1 μg/mL) for 16 h. Then, the cells were stained with anti-cholera toxin B subunit and anti-DR4 antibody for 1 h, and then incubated with Alexa Fluor 568-conjugated antibody for 45 min. Finally, the cells were analyzed by confocal fluorescence microscopy. Original magnification, ×63. (B) MGC803 cells were treated with under similar conditions to those in (A). The cells were then fractionated by the ultracentrifuge. Locations of lipid rafts (fractions 1–2) were determined using caveolin-1. The indicated proteins were analyzed by Western blot.

3.2. MβCD partially prevents cetuximab/TRAIL-induced DR4 clustering and DISC formation in lipid rafts

Because we have previously shown that DR4 clustering and DISC formation in lipid rafts occurs in gastric cancer cells treated with the combination of cetuximab and TRAIL, we next wanted to identify whether MβCD, a cholesterol-depletion agent that disrupts lipid rafts, interferes with this process. As shown in Fig. 2A, exposure to MβCD alone did not affect lipid raft aggregation or DR4 clustering in MGC803 cells. Interestingly, pre-incubation of cells with 2.5 mg/mL MβCD for 30 min partially reversed cetuximab and TRAIL-induced lipid raft aggregation and DR4 clustering. In addition, MβCD did not significantly influence the constituents of DISC in the lipid raft fractions (1 and 2, Fig. 2B). However, pre-incubation of cells with MβCD partially prevented cetuximab/TRAIL-induced translocation of DR4 and FADD into lipid raft fractions (1 and 2, Fig. 2B) as well as the cleavage of caspase-8 (Fig. 2B). Together, the results indicate that maintenance of the integrity of lipid rafts is necessary for DISC formation in gastric cancer cells.

3.3. MβCD partially reverses apoptosis induced by cetuximab and TRAIL in gastric cancer cells

To further determine the involvement of DISC in apoptosis induced by the combined treatment with cetuximab and TRAIL, MGC803 and SGC7901 cells were pre-incubated with 2.5 mg/mL MβCD for 30 min followed by treatment with the cetuximab/TRAIL combination for 24 h. MβCD alone did not obviously affect cell apoptosis (Fig. 3A). Compared with the effects of incubation of cetuximab and TRAIL alone, pre-incubation with MβCD partially suppressed apoptosis ($19.15 \pm 3.89\%$ *vs.* $10.17 \pm 3.02\%$ and $16.09 \pm 3.51\%$ *vs.* $8.92 \pm 2.67\%$ for MGC803 and SGC7901, respectively, $P < 0.05$, Fig. 3A). Moreover, PARP cleavage and caspase-3

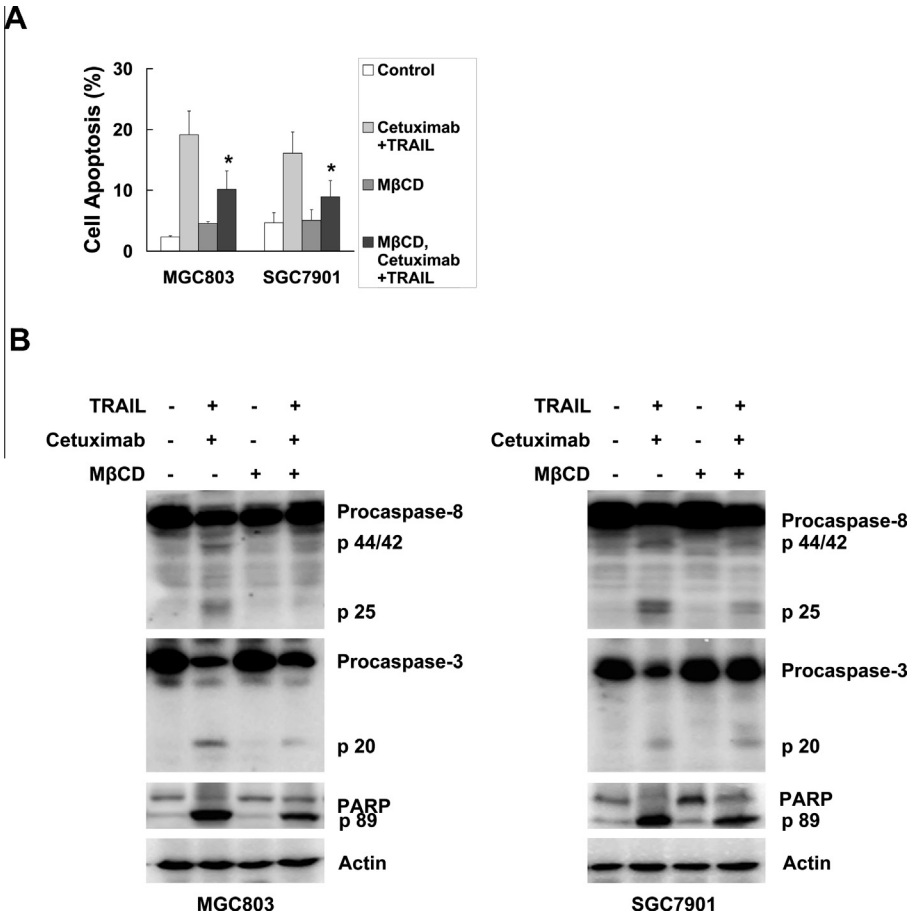

Fig. 3. MβCD partially reduced apoptosis induced by cetuximab and TRAIL in gastric cancer cells. (A) MGC803 and SGC7901 cells were treated exposed to 2.5 mg/mL MβCD for 30 min in serum-free medium. The cells were then washed with PBS, resuspended in complete culture medium and treated with TRAIL (100 ng/mL) and cetuximab (1 μg/mL) for 24 h. The percentage of apoptotic cells was quantitated by flow cytometry. ∗Preincubated with MβCD before TRAIL and cetuximab *vs.* incubated with TRAIL and cetuximab alone, $P < 0.05$. (B) MGC803 and SGC7901 cells were treated under similar conditions to those in (A). The expression of caspase-3, caspase-8, and PARP was analyzed by Western blot.

and caspase-8 activation were also partially prevented (Fig. 3B). These results suggest that lipid rafts provide a vital platform for DISC formation and subsequent apoptosis in gastric cancer cells.

4. Discussion

Death receptor-mediated pathways of apoptosis include TRAIL and the FasL pathway. Both TRAIL and FasL depend on DISC formation, which is the most upstream event of apoptosis [17]. Lipid rafts provide a membrane platform for DISC formation which plays an important role in transmitting TRAIL and FasL death signals [18,19]. In multiple myeloma cells, death receptor recruitment and DISC formation in lipid rafts, induced by edelfosine, has been shown to potentiate TRAIL and FasL-mediated cell killing [20]. While disruption of membrane raft domains with MβCD interferes with edelfosine-induced DISC assembly and apoptosis in Jurkat cells [21]. Moreover, apoptotic signaling molecule-enriched rafts also play a key role in quercetin-induced DISC formation and apoptosis in colon cancer cells [22]. In the present study, although TRAIL alone induced some DR4 and FADD translocation into lipid rafts in TRAIL-resistant gastric cancer cells, no obvious DR4 clustering or caspase-8 cleavage was detected. The inability of TRAIL to induce sufficient lipid raft aggregation and subsequent recruitment of DISC constituents may be one explanation for TRAIL resistance in gastric cancer cells. Accordingly, the regulation of DISC assembly in lipid rafts probably increases the sensitivity of gastric cancer cells to TRAIL.

Recent studies suggest that the cross-talk between different receptors regulates their activity [23]. In clinical treatment, the combination of the anti-EGFR antibody cetuximab and chemotherapy has obtained remarkable curative effects in KRAS wild-type colorectal cancer patients [24]. The mechanism of tumor growth inhibition is thought to involve cetuximab binding to EGFR and the subsequent blockade of EGFR-mediated proliferation pathways. Since EGFR is located in lipid rafts [25], and these are the membrane platforms for interactions between different receptors, the blockade of EGFR may also indirectly influence other receptor signals. With regard to the effect of cetuximab on the TRAIL pathway, Van et al. has reported that TRAIL activates EGFR, and that cetuximab-mediated sensitization to TRAIL is due to the inhibition of TRAIL-mediated EGFR activation in colon cancer cells [26]. Recently, it has been shown that phosphoinositide 3 kinase (PI3K) is also associated with lipid rafts, which provide a platform for the interactions of EGFR, c-Src, and PI3K, which in turn leads to the activation of cellular survival signaling in breast cancer cells [27]. In addition, the PI3K inhibitor LY303511 has been shown to amplify TRAIL-induced apoptosis in cervical cancer cells by enhancing DR5 oligomerization and DISC assembly [28]. However, whether the interaction between EGFR and death receptors affects death receptor-mediated DISC formation in lipid rafts is poorly understood. Surprisingly, distinct from cetuximab-mediated inactivation of EGFR pathway as shown in our previous studies [14], cetuximab alone also induced DR4 clustering and the translocation of DR4 and FADD into lipid rafts without subsequent caspase-8

cleavage and apoptosis. However, cetuximab combined with TRAIL significantly induced DISC formation, caspase-8 cleavage and increased TRAIL-induced apoptosis in gastric cancer cells. Meanwhile, the cholesterol-depletion agent MβCD partially prevented DR4 clustering and DISC formation, and reversed apoptosis induced by the cetuximab and TRAIL combination. These results indicate that although cetuximab alone does not induce apoptosis, it contributes to the recruitment of DISC constituents to lipid rafts and thereby provides favorable conditions for TRAIL-triggered apoptosis. Thus DR4 recruitment and DISC formation in lipid rafts could explain cetuximab-mediated sensitization to TRAIL in gastric cancer cells.

Taken together, the promotion of DISC formation in lipid rafts may be a novel mechanism by which cetuximab increases TRAIL sensitivity. Our results provide evidence that cetuximab plus TRAIL could improve the therapeutic effect in gastric cancer. Moreover, our findings suggest that the regulation of DISC assembly in lipid rafts could be an appealing target for intervention in gastric cancer.

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Conflict of interest statement

None declared.

Author contributions

L.X. contributed to acquisition of data, manuscript preparation. X.J.H., K.Z.H. and H.C.Z. contributed to analysis and interpretation of data. X.J.Q. contributed to draft the article and revise it critically for important intellectual content. Y.P.L. contributed to design of the study, final approval of the version to be submitted.

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