

Quercetin enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts

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Abstract

Cytokines such as tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) can induce apoptosis in colon cancer cells through engagement of death receptors. Nevertheless, evading apoptosis induced by anticancer drugs characterizes many types of cancers. This results in the need for combination therapy. In this study, we have investigated whether the flavonoid quercetin could sensitize human colon adenocarcinoma cell lines to TRAIL-induced apoptosis. We report that quercetin enhanced TRAIL-induced apoptosis by causing the redistribution of DR4 and DR5 into lipid rafts. Nystatin, a cholesterol-sequestering agent, prevented quercetin-induced clustering of death receptors and sensitization to TRAIL-induced apoptosis in colon adenocarcinoma cells. In addition, our experiments show that quercetin, in combination with TRAIL, triggered the mitochondrial-dependent death pathway, as shown by Bid cleavage and the release of cytochrome *c* to the cytosol. Together, our findings propose that quercetin, through its ability to redistribute death receptors at the cell surface, facilitates death-inducing signaling complex formation and activation of caspases in response to death receptor stimulation. Based on these results, this study provides a

challenging approach to enhance the efficiency of TRAIL-based therapies. [Mol Cancer Ther 2007;6(9):2591–9]

Introduction

Cancer is the result of a stepwise, progressive disruption of cellular signaling cascades, which control cell proliferation, survival, and differentiation (1). Despite aggressive therapies, most common cancers do not easily undergo apoptosis and they resist to chemotherapy. These result in the need for drug combinations. The target is to reactivate apoptotic cascades and render the cells apoptosis prone so that low doses of cytotoxic drugs cause apoptosis.

Several food polyphenols may act as chemopreventers by reducing the incidence of many types of cancers, especially in colon epithelia (2). Quercetin (3,3',4',5,7-pentahydroxyflavone) is a flavonoid ubiquitous in nature, described as a potential anticancer agent (3, 4). We have shown recently that quercetin mediates preferential degradation of oncogenic Ras proteins, proposing that it could act as a chemopreventer for cancers with frequent mutations of RAS genes (5). Other studies have shown that quercetin acts as a sensitizer in CD95-mediated apoptosis (6) and enhances tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–mediated apoptosis through Akt dephosphorylation (7).

TRAIL is one of the members of the tumor necrosis factor gene superfamily. It triggers apoptosis by binding as a homotrimeric subunit structure (8) to death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2), which contain a death domain in their cytoplasmic tail (9). TRAIL also binds to the decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4) that cannot transmit a cell death signal. Activation of the death signaling pathway involves the recruitment of the adapter protein Fas-associated death domain (FADD) to the DR4 and DR5 and the subsequent recruitment of procaspase-8 or procaspase-10 in a death-inducing signaling complex (DISC; refs. 10, 11). This leads to caspase-8 cleavage and activation of two signaling pathways (12). In type I cells, caspase-8 directly activates effector caspases leading to apoptosis. In type II cells, caspase-8 causes the cleavage of Bid into its active form t-Bid, which translocates to the outer mitochondrial membrane and induces cytochrome *c* and Smac/DIABLO release to the cytoplasm and caspase-9 activation (13). TRAIL has gained interest as a potential candidate for cancer therapy mainly due to its ability to selectively induce apoptosis in many transformed cells but not in normal cells (14), a fact that has been explained by the percentage of large numbers of DR4 and DR5 on the surface of tumor cells and the decoy receptors on normal cells, respectively (15).

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Lipid rafts are plasma membrane microdomains enriched in cholesterol and glycosphingolipids. They have an important role in clustering or aggregating surface receptors into membrane complexes at specific sites and this is essential for initiating signaling from several receptors (16–18).

In HT-29 colon adenocarcinoma cells, quercetin does not induce apoptosis in relatively low concentrations. On the other hand, these cells have already been described as resilient to apoptosis induced by cytokines, such as TRAIL and Fas-L, (19) and this is correlated to their death receptors levels (20). Our results show that, although quercetin does not increase the expression of death receptors at the surface of the cells, it sensitizes colon adenocarcinoma cells to TRAIL-induced apoptosis, mainly through its ability to aggregate death receptors into lipid rafts and thus facilitating the DISC formation as well as the activation of the mitochondrial-dependent death pathway. These findings propose that quercetin could act as a sensitizer for TRAIL-induced apoptosis and this may be a novel strategy for the treatment of a variety of human tumors that resist therapies based on TRAIL.

Materials and Methods

Cell Cultures, Cytotoxicity, and Apoptosis Assays

HT-29, SW-620, and Caco-2 cells (American Type Culture Collection, University Boulevard, Manassas, VA) were cultured in DMEM supplemented with 10% fetal bovine serum, antibiotics, and nonessential amino acids (Invitrogen Life Technologies).

For cell viability, cells were treated with quercetin (Sigma) and/or TRAIL (21) and/or nystatin. All samples were prepared in triplicate. Cells were fixed with methanol, stained with 0.5% crystal violet, and washed with PBS and the remaining crystal violet was extracted using 30% acetic acid. Absorbance was measured at 595 nm. The percentages of apoptotic cells were assessed by exposure to the DNA binding dye Hoechst 33342 (Sigma) and apoptotic measurements were carried out under a fluorescent inverted microscope (Nikon Eclipse T-200). SD was used for error bar generation. Differences were evaluated using Student's *t* test. *P* < 0.05 was considered significant.

Reverse Transcription-PCR

RNA isolation was done using the Trizol reagent (Invitrogen Life Technologies). cDNA was prepared using an oligo(dT) primer and Moloney leukemia virus reverse transcriptase (Promega) following standard protocols. Primers used were the following: DR4, 5'-CAGAACGTCCTGGAGCCTGTAAC-3' (forward) and 5'-ATGCCATGGCCTGATTCTTGTG-3' (reverse); DR5, 5'-GGGAAGAAGATTCTCTGAGATGTG-3' (forward) and 5'-ACATTGTCCTCAGCCCCAGGTCG-3' (reverse); and glyceraldehyde-3-phosphate dehydrogenase, 5'-ACCA-CAGTCCATGCCATCAC-3' (forward) and 5'-TCCAC-CACCCTGTTGCTGTA-3' (reverse). The cDNA product was amplified by PCR with Taq DNA polymerase (Promega) using standard protocols; the annealing temperature was

58°C for all primers. The amplified products were separated on 1.2% agarose gels, stained with ethidium bromide, and photographed using UV illumination. Intensity values were measured using Molecular Dynamics ImageQuant software (Amersham Biosciences). PCR products were normalized to glyceraldehyde-3-phosphate dehydrogenase expression.

Immunoblotting

Whole-cell lysates prepared with lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 250 mmol/L sucrose, 1 mmol/L EDTA, 10 mmol/L NaF, 1 mmol/L EGTA, and 1% Triton X-100 supplemented with protease and phosphatase inhibitors were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Immunoblotting was done with the following antibodies: poly(ADP-ribose) polymerase, Bid, Bax, cytochrome *c*, caveolin-1, caspase-3, caspase-8, and α -tubulin (Santa Cruz Biotechnology, Inc.); FADD (BD Transduction Laboratories); DR4 and DR5 (Alexis Biochemicals); and anti-ATP synthase (Invitrogen). Secondary antibodies were from Jackson. The membranes were scanned with Image Storm scanner (Molecular Dynamics) and the values were measured using Molecular Dynamics ImageQuant software (Amersham Biosciences). Ponceau staining was used for protein loading control, and extracellular signal-regulated kinase-2 (Santa Cruz Biotechnology) was used for normalization.

Immunofluorescence Microscopy

Treated cultures were fixed in 4% PBS-buffered paraformaldehyde for 20 min, washed in PBS, and quenched with 50 mmol/L NH₄Cl for 15 min. Blocking was in PBS containing 5% fetal bovine serum. The cells were incubated with primary antibodies against DR4 (clone 02B4), DR5 (clone 5C11/G8; prepared in the laboratory of Cell Signaling and Apoptosis), and caveolin-1 (Santa Cruz Biotechnology). Secondary antibodies antirabbit red (A21428) and antimouse green (A11001) were from Alexis Biochemicals. The cells were analyzed by fluorescence microscopy using an inverted microscope equipped with a filter system. Images were obtained using a fluorescent inverted microscope (Nikon Eclipse T-200) with a digital camera.

Raft Isolation

Raft isolation was done as described (18). Briefly, the cells (100×10^6) were washed with ice-cold PBS and lysed in MES buffer (Sigma) supplemented with 150 mmol/L NaCl and protease inhibitors, containing 1% Triton X-100, before passing through an ice-cold cylinder cell homogenizer. Then, lysates were diluted with MES buffer containing 80% sucrose (w/v), placed at the bottom of a linear sucrose gradient, and centrifuged at 39,000 rpm for 20 h at 4°C. One-milliliter fractions were collected from the top of the gradient, subjected to SDS-PAGE, and immunoblotted.

Immunoprecipitation and Analysis of DISC

The ligand affinity precipitation was done using biotinylated TRAIL (Bio-TRAIL) in combination with streptavidin beads (Pierce Biotechnology), as described previously (22). Ligand affinity precipitates were washed with lysis buffer

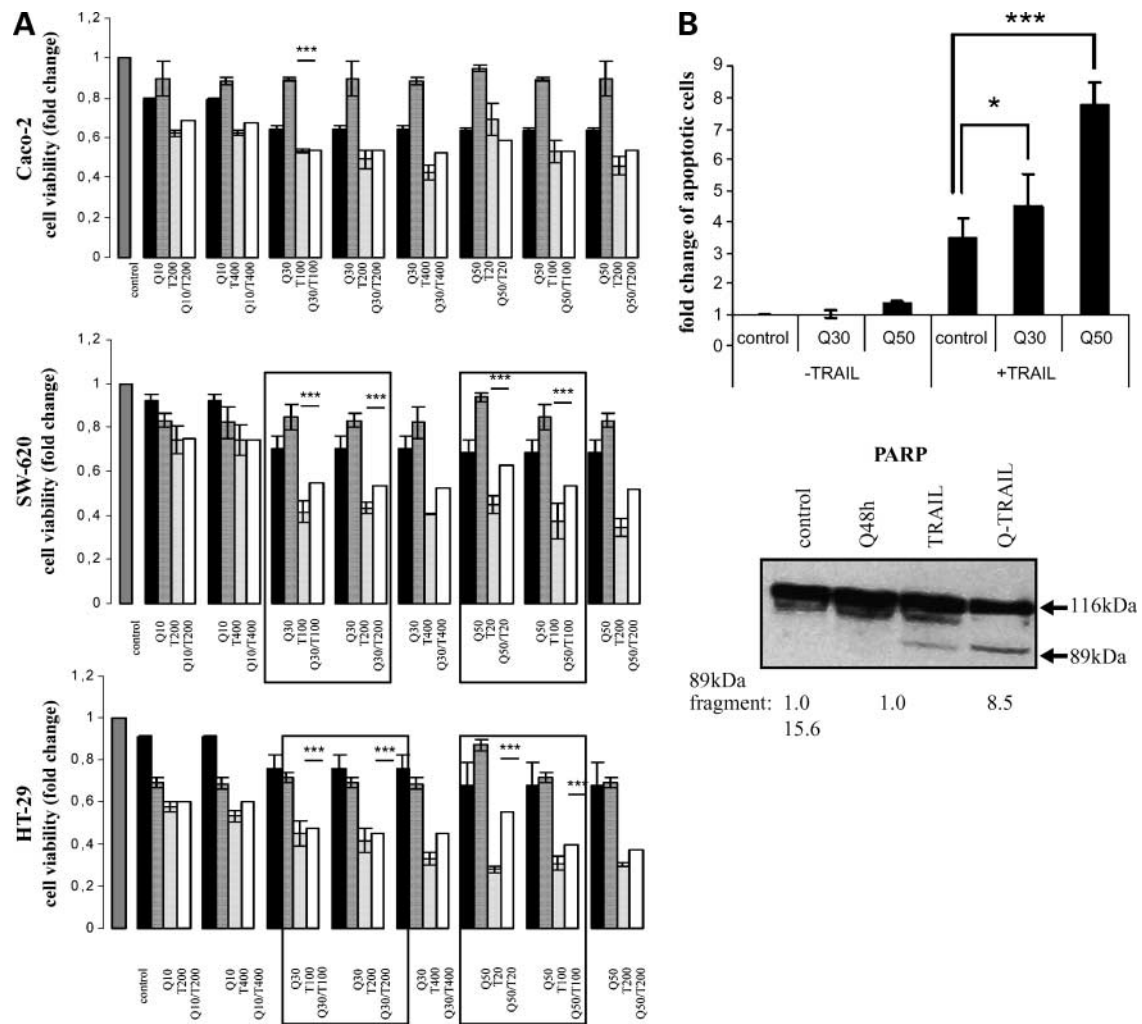


Figure 1. Quercetin sensitizes colon cells to TRAIL-induced apoptosis. **A**, Caco-2 (top), SW-620 (middle), and HT-29 (bottom) cells were either left untreated (control) or treated with the indicated conditions of quercetin (48 h; *black columns*), TRAIL (24 h; *hatched columns*), or the combination of the two (24-h quercetin plus 24-h cotreatment with quercetin-TRAIL; *dotted columns*) and the percentage of cell viability was measured by crystal violet staining as described in Materials and Methods. Cell viability is presented as fold change of the absorbance of treated/untreated cells, for each condition. *Columns*, cell viability; *bars*, SD. Most important combinations are highlighted into frames. **B**, *left*, following incubation with 30 or 50 $\mu\text{mol/L}$ quercetin for 24 h, the cells were treated or not with 100 ng/mL TRAIL for additional 6 h and the percentage of apoptotic cells was estimated by Hoechst 33258 staining; *right*, Western blot analysis of the 116-kDa poly(ADP-ribose) polymerase (PARP) polymerase and its 89-kDa cleaved fragment. *Lane 1*, untreated cells; *lane 2*, quercetin for 24 h plus overnight incubation; *lane 3*, TRAIL overnight; *lane 4*, quercetin for 24 h plus overnight incubation with quercetin and TRAIL together.

and the protein complexes were eluted from the beads by the addition of SDS sample buffer and heating at 42°C for 15 min. Proteins were separated in SDS-PAGE and immunoblotted.

Cell Fractionation

Mitochondrial and cytosolic fractions were prepared as described (19). Cells were resuspended in lysis buffer before passing through an ice-cold cylinder homogenizer. Unlysed cells and nuclei were spun down at $750 \times g$ at 4°C for 10 min. The supernatant was centrifuged at $10,000 \times g$ at 4°C for 25 min; the pellet was resuspended in lysis buffer and represents the mitochondrial fraction. The supernatant was spun at $10,000 \times g$ for 1 h and the final supernatant represents the cytosolic fraction.

Flow Cytometry Analysis

For the histologic analysis of apoptosis, the cells were washed with PBS containing 0.1% Tween and fixed with cold (-20°C) methanol for 30 min, and after washing, a fluorescein-conjugated mouse monoclonal antibody (clone M30, CytoDEATH, Roche) for cleaved cytokeratin-18 was added for 1 h at room temperature. The cells were washed and subjected to fluorescence-activated cell sorting analysis.

Results

Quercetin and TRAIL Cooperate to Induce Apoptosis in Colon Adenocarcinoma Cell Lines

To study the effect of the polyphenol quercetin in TRAIL-induced apoptosis, we used the Caco-2 intermediate

adenoma as well as the SW-60 and HT-29 adenocarcinoma cell lines. In Fig. 1A, the cells were treated with quercetin (48 h) or TRAIL (24 h) or the combination of the two (24-h quercetin for 24 h plus 24-h coincubation with quercetin-TRAIL). Quercetin seemed to be a potent sensitizer to TRAIL-induced apoptosis, especially for SW-620 and HT-29 cells. More specifically, in almost all cases, the combination of the two agents caused reduction of the cell viability, which was more than additive (Fig. 1A, *white columns*). The most significant combinations, using relatively low concentrations of both agents, are highlighted into frames. No significant differences were observed in Caco-2 cells, whereas the combinatory effects of quercetin and TRAIL were similar to additive. The apoptotic potential of the combination of quercetin-TRAIL was specifically studied on HT-29 colon adenocarcinoma cells, which have shown limited sensitivity to death receptor-mediated apoptosis. Apoptosis was estimated by measuring the percentage of apoptotic nuclei as identified by Hoechst 33258 staining. For this, the cells were pretreated with quercetin for 24 h and subjected to

quercetin-TRAIL treatment for the last 6 h. The apoptotic effect under cotreatment conditions was higher than additive, as indicated in Fig. 1B (*left*). This was further confirmed by the enhanced poly(ADP-ribose) polymerase cleavage under cotreatment conditions. The levels of the 89-kDa cleaved fragment of poly(ADP-ribose) polymerase were doubled when the cells were pretreated for 24 h with quercetin before overnight (16 h) incubation with TRAIL compared with cells that were treated with TRAIL without preincubation with the polyphenol (Fig. 1B, *right*). Notably, quercetin does not induce poly(ADP-ribose) polymerase cleavage at the concentration used and this also confirms the low levels of apoptotic cells observed by Hoechst. All these data show that quercetin enhances TRAIL efficiency to induce apoptosis in colon adenocarcinoma cells.

Sensitization of HT-29 Cells by Quercetin- to TRAIL-Induced Cell Death Is Not Correlated to Changes in Death Receptor Expression

Analysis of death receptor stimulation was the next step. Reverse transcription-PCR analysis for both TRAIL

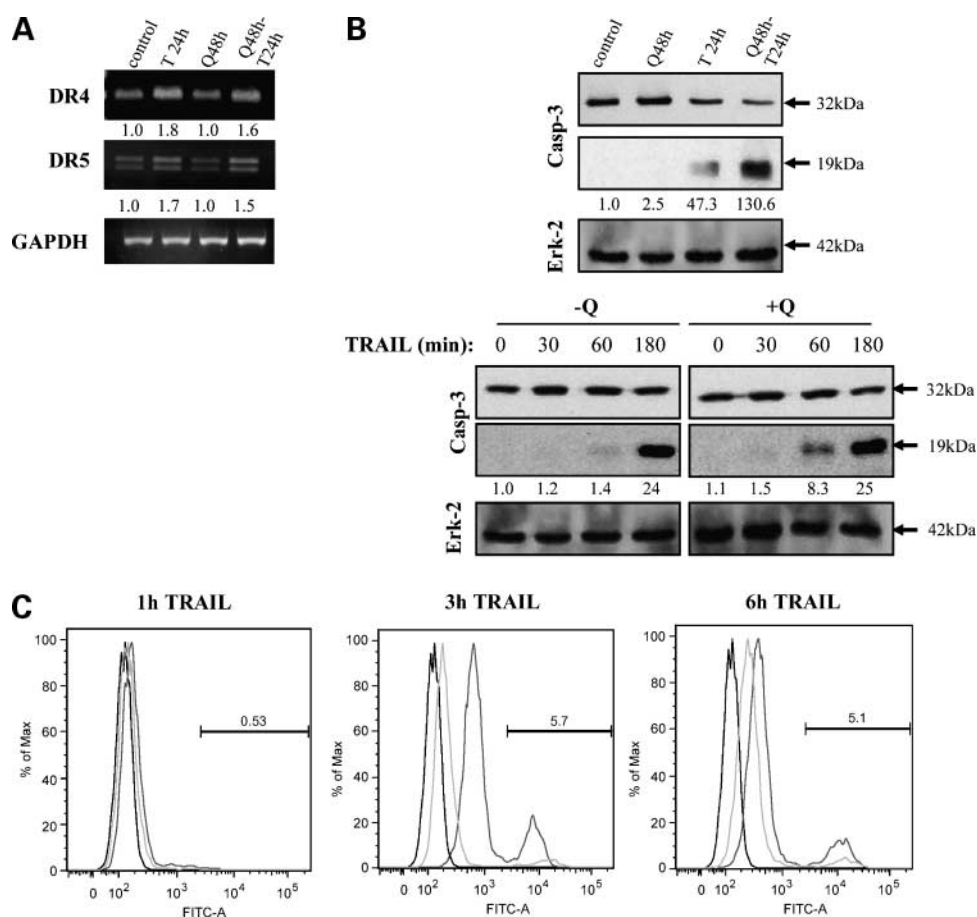
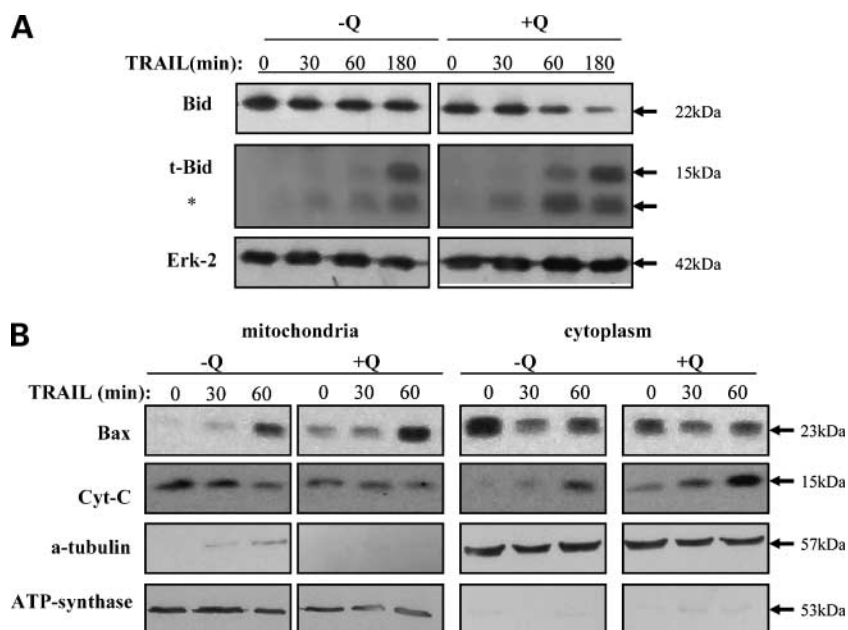


Figure 2. Quercetin enhances TRAIL-induced caspase-3 cleavage. **A**, reverse transcription-PCR analysis of death receptors after treatment with 100 ng/mL TRAIL for 24 h, 30 μ mol/L quercetin for 48 h, or the combination of the two (24-h quercetin before cotreatment with TRAIL for additional 24 h). *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. **B**, *top*, Western blot analysis of total caspase-3 (*Casp3*) and its proteolytic 19-kDa fragment at similar conditions as in (**A**), and extracellular signal-regulated kinase-2 (*Erk-2*) was used as a loading control. **C**, fluorescence-activated cell sorting analysis of cytochrome c using a FITC-conjugated antibody. The cells were pretreated or not with 30 μ mol/L quercetin for 24 h before the addition of 100 ng/mL TRAIL for the last 1, 3, and 6 h. *Black line*, untreated cells; *light gray line*, cells treated with TRAIL; *dark gray line*, cells treated with TRAIL after pretreatment with quercetin.

Figure 3. Quercetin induces mitochondrial changes. **A**, cells were stimulated with 100 ng/mL TRAIL for the indicated times following or not 24-h preincubation with 30 μ mol/L quercetin for 24 h. Total protein extracts were prepared and Western blot analysis was done using an antibody that recognize both total and cleaved forms of Bid. The lower band representing partial Bid degradation product is indicated by an asterisk (*). **B**, mitochondrial and cytoplasmic extracts were prepared as described in Materials and Methods after pretreatment or not of HT-29 cells with quercetin, followed by incubation with TRAIL at the indicated conditions. The levels of Bax and cytochrome *c* (Cyt-C) in the mitochondria and the cytosol were studied by immunoblotting. The α -tubulin and the ATP synthase antibodies were used as cytoplasmic and mitochondrial markers, respectively.



receptors DR4 and DR5 showed that TRAIL enhanced both DR4 and DR5 expression, but no synergism with quercetin on mRNA levels was observed. The data were further confirmed by Western blot analysis for DR4 and DR5 under the same conditions (data not shown), indicating that quercetin alone or in combination with TRAIL does not affect death receptor expression.

We next examined the effect of quercetin in combination with TRAIL on the effector caspase-3 cleavage. Interestingly, the 19-kDa cleaved form of caspase-3 after TRAIL treatment was stronger (\sim 3-fold) when the cells were pretreated with quercetin for 24 h (Fig. 2B, *top*). Time course analysis of caspase-3 cleavage showed that pretreatment with quercetin induced stronger cleavage of caspase-3 and at an earlier time point (Fig. 2B, *bottom*). Treatment with quercetin alone did not induce cleavage of caspase-3, confirming the weak ability of the compound to induce apoptosis in HT-29 cells at this concentration. Given that cytokeratin is a direct substrate for caspase-3 and a strong marker for caspase-3 activity in epithelial cells, fluorescence-activated cell sorting analysis was done using a cytokeratin-18 FITC-conjugated antibody, which binds only to cleaved cytokeratin. Cells pretreated with quercetin for 24 h had higher levels of cleaved cytokeratin after treatment with TRAIL for 1, 3, and 6 h (Fig. 2C). Quercetin alone did not induce significant changes in cleaved cytokeratin-18 levels (data not shown). The results were further confirmed by Annexin V-FITC staining at the same conditions (data not shown).

The Mitochondrial Pathway Contributes to Quercetin-Enhanced TRAIL-Mediated Apoptosis

The observation that TRAIL-mediated cleavage of caspase-3 was facilitated by quercetin led to the question if this was a result of quercetin effect at the level of DISC formation and/or due to mitochondrial apoptotic path-

way changes induced by quercetin. In Fig. 3A, it is shown that TRAIL-induced cleavage of the Bid protein to its truncated form t-Bid was facilitated by pretreatment with quercetin.

To further identify the involvement of mitochondria in quercetin-facilitated apoptosis, we estimated Bax and cytochrome *c* redistributions in cells exposed to quercetin-TRAIL combination. In Fig. 3B, the cells were incubated for 24 h with medium alone or with 30 μ mol/L quercetin before coincubation with TRAIL for 30 or 60 min. After cell fractionation, it was observed that cytochrome *c* release from the mitochondria to the cytoplasm was slightly induced by quercetin, whereas the effect was stronger under cotreatment conditions. Bax distribution to the mitochondria mediated by TRAIL after 30- or 60-min incubation was strongly enhanced by quercetin.

Quercetin Enhances DISC Formation

As it was shown earlier in this study, quercetin treatment of HT-29 cells did not induce significant changes in the expression of TRAIL receptors DR4 and DR5. In addition, fluorescence-activated cell sorting analysis showed that quercetin did not change the cell surface levels of death receptors (data not shown). These observations did not rule out the possibility that quercetin could facilitate the formation of the DISC complex in response to TRAIL stimulation. Treatment of the cells with biotin-conjugated TRAIL (Bio-TRAIL) for 3 min and pulldown of the DISC-containing receptors with streptavidin beads indicated that caspase-8 and FADD are recruited to the DISC (Fig. 4A). In Fig. 4B, Bio-TRAIL was added to the cells after 24-h pretreatment with quercetin for the indicated times. Quercetin enhanced the TRAIL-induced formation of DR4 and DR5 aggregates and the recruitment of the procaspase-8 and the adapter protein FADD to the complex. More specifically, the ratio of the 20 min/1 min levels of DR4 and

DR5 was elevated in pretreated cells compared with those that were not subjected to quercetin treatment. Similar results were obtained for caspase-8 and FADD, suggesting that the entire complex formation was enhanced by quercetin.

Quercetin Induces the Redistribution of TRAIL Receptors in Lipid Rafts

Recent studies indicating the role of plasma membrane microdomains enriched in cholesterol and glycosphingolipids in the initiation of death receptor-induced cell death led us to investigate whether quercetin influences TRAIL receptor redistribution in the plasma membrane. To this end, untreated and treated with quercetin for 48-h cells were subjected to ultracentrifugation onto a linear sucrose gradient and cell fractions were isolated. Treatment of HT-29 cells with 30 $\mu\text{mol/L}$ quercetin resulted in the redistribution of DR4, DR5, and the adapter protein FADD in the fractions 4, 5, and 6 that express the raft-associated protein caveolin-1. Procaspase-8, but not caspase-3 (negative control), was also present in these fractions (Fig. 5A). To further verify the quercetin-induced clustering of DR4 and DR5 on the cell membrane, we did fluorescence microscopy using the antibodies against DR4, DR5, and caveolin-1. In control cells, DR4 (Fig. 5B, *top*) and DR5 (Fig. 5B, *bottom*) showed a diffused distribution; quercetin

treatment for 48 h induced the clustering of the death receptors at the cell surface. Furthermore, both DR4 and DR5 were colocalized with the raf-associated protein caveolin-1 after treatment with quercetin (Fig. 5B, *overlay*).

The cholesterol-sequestering molecule nystatin suppressed quercetin-induced changes in the cell membrane and reduced death receptor clustering. Quercetin was added to the cells for 48 h and nystatin was added only for the last 12 h, to avoid extended cytotoxicity induced by the molecule (Fig. 5B, *bottom*). Notably, nystatin partially reduced the effect of quercetin on TRAIL-induced apoptosis in HT-29 cells (Fig. 5C). These findings indicate that quercetin triggers the clustering of death receptors into specific plasma membrane microdomains and this facilitates TRAIL-induced apoptosis.

Discussion

One strategy to overcome resistance of tumor cells is the combination of drugs that activate apoptotic pathways and natural products that facilitate apoptosis in resistant cancer cells (23, 24). Ideally, the use of low doses of a compound that reactivate apoptotic cascades and render the cancer cells apoptosis prone would reduce the need for high doses of the drug (25).

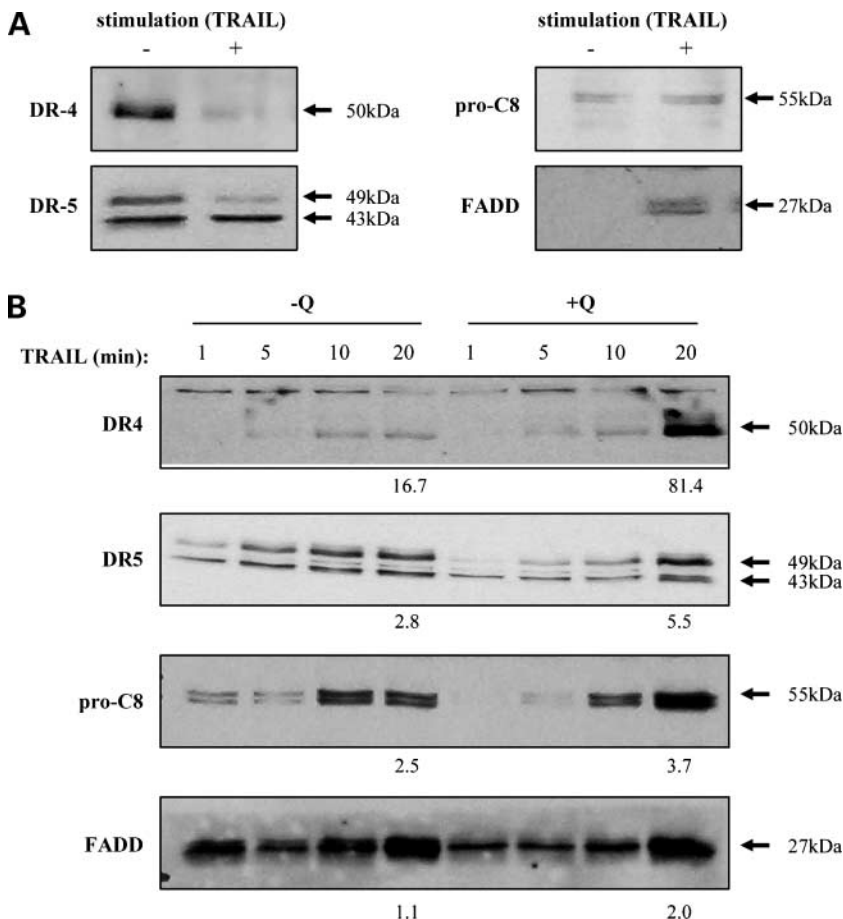


Figure 4. Quercetin enhances the DISC formation. **A**, the cells were left untreated (-) or treated with 100 ng/mL Bio-TRAIL (+) for 3 min and, after lysis, Bio-TRAIL was added to lysates from the unstimulated cells. DISC complex was precipitated using streptavidin beads. The functional proteins that were bound to Bio-TRAIL were analyzed by Western blot. **B**, HT-29 cells were left untreated or treated with 30 $\mu\text{mol/L}$ quercetin for 48 h before exposure to 100 ng/mL of Bio-TRAIL for the indicated times. Following incubation and immunoprecipitation of DISC with streptavidin beads, the samples were immunoblotted for the indicated proteins. *Pro-C8*, procaspase-8.

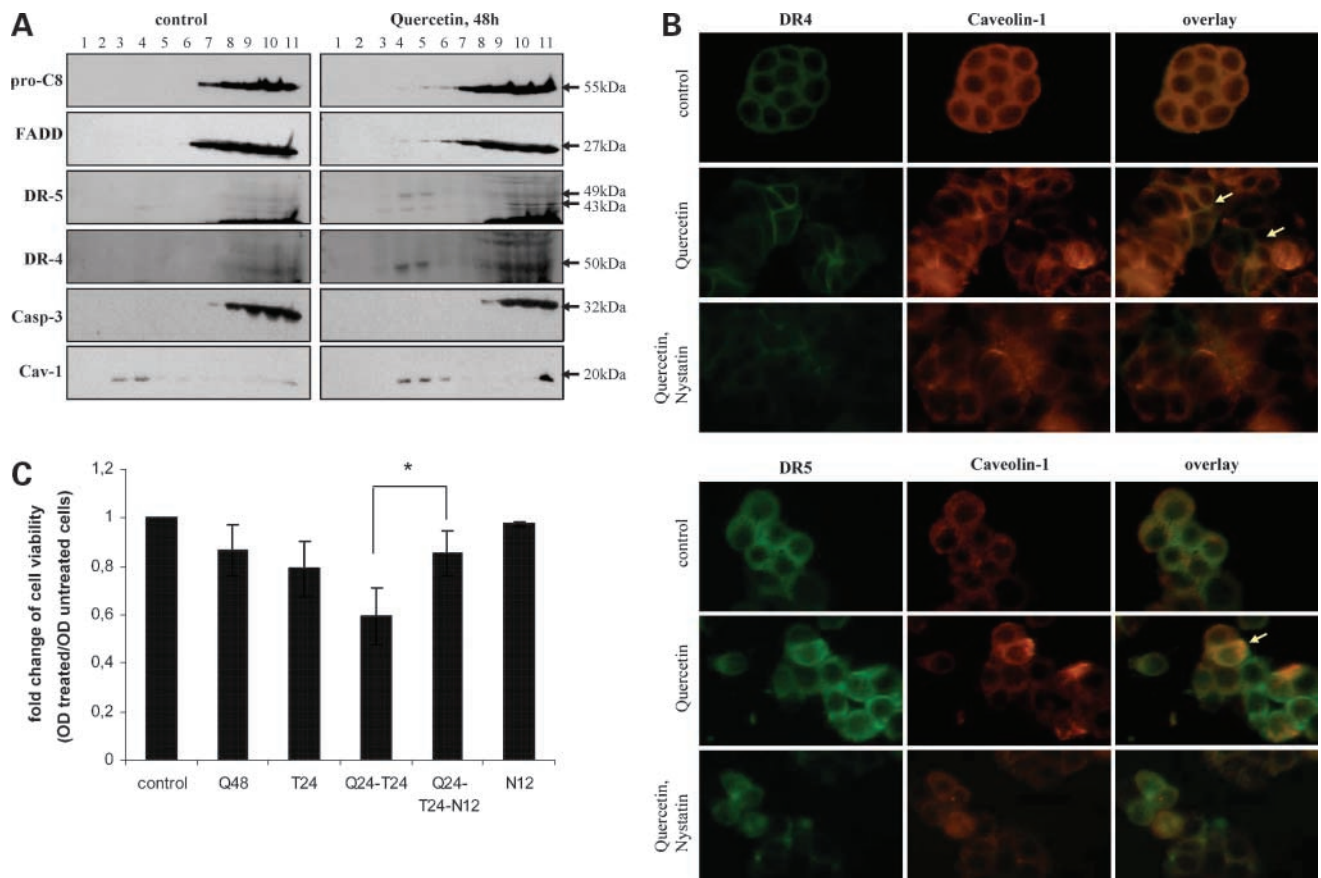


Figure 5. Treatment of HT-29 cells with quercetin triggers the redistribution of DR4 and DR5 in lipid rafts. **A**, HT-29 cells were left untreated (*left*) or treated (*right*) with 30 $\mu\text{mol/L}$ quercetin for 48 h and, after separation in a sucrose gradient, subjected to immunoblot analysis for the indicated proteins. **B**, fluorescence microscopy of cells treated or not with 30 $\mu\text{mol/L}$ quercetin for 48 h and stained with antibodies against DR4 (*top*), DR5 (*bottom*), and caveolin-1. Cells were treated with 20 ng/mL nystatin for the last 12 h (*bottom*). Representative images. Original magnification, $\times 40$. **C**, HT-29 cells were left untreated or treated with either quercetin (30 $\mu\text{mol/L}$, 48 h) or TRAIL (100 ng/mL, 24 h) or their combination (as in Fig. 2A), or nystatin (20 ng/mL, for the last 12 h) and viability assay with crystal violet staining was done. *Columns*, fold change of cell viability; *bars*, SD. *Cav-1*, caveolin-1.

Our study, as well as most other combination studies that involve natural compounds, serves the purpose of stimulating research toward specific natural compounds that can be used as the base structure to design chemopreventive/sensitizing agents. The concentrations of quercetin used in our experiments are not dramatically higher than the concentrations that could be achieved in the plasma after oral administration of dietary supplements or indeed dietary components (26, 27).

TRAIL is a promising therapeutic agent that induces apoptosis selectively in cancer cells. TRAIL has shown limited efficiency in the treatment of several colon cancer cells because of intrinsic or acquired resistance (28, 29). Interestingly, our observations suggest that quercetin cooperate with TRAIL to induce apoptosis in HT-29 cells through its ability to redistribute TRAIL receptors and other components of the DISC complex into lipid rafts. This facilitates the formation of the DISC and the downstream signaling pathway, which contributes to Bax conformational changes, release of cytochrome *c*, and apoptosis.

Pretreatment with Quercetin Enhances TRAIL-Induced Caspase-3 and Bid Cleavage

In colon adenocarcinoma cell lines tested, quercetin was found to enhance TRAIL-induced cell death, whereas the effects of cotreatment were not so significant for intermediate adenoma cells, indicating a different cell response, probably dependent on the stage they represent. Pretreatment of HT-29 colon adenocarcinoma cells with quercetin also increased the percentage of apoptotic cells after TRAIL addition. These data show that the resilient to TRAIL HT-29 cells could be converted to TRAIL-sensitive cells by pretreatment with quercetin. Quercetin has been shown to activate apoptotic pathways and to sensitize cells to death receptor-induced apoptosis (6, 30). In a recent study, it has been proposed that 200 $\mu\text{mol/L}$ quercetin induce Akt dephosphorylation and by this way enhances TRAIL-induced cytotoxicity by caspase activation in prostate cancer cells (7). Nevertheless, in our experimental setup, where the highest concentration of quercetin used was 30 $\mu\text{mol/L}$, there were no changes in phosphorylated Akt levels.

The effector caspase-3 activation has been shown to be required for TRAIL-induced apoptosis (11) in response to TRAIL exposure. In the conditions tested here, pretreatment with quercetin followed by TRAIL incubation induced higher levels of caspase-3 cleavage, which occurred faster and was accompanied by cleavage of cytokeratin-18. Interestingly, our experiments showed that the combination of these two agents also resulted in accelerated cleavage of Bid into its active form t-Bid, followed by stronger cytochrome *c* release to the cytosol, as well as aggregation of Bax to the mitochondria. These processes gain a lot of interest in the field of mitochondria targeting for cancer chemoprevention (31), whereas many putative cancer chemopreventive agents possess the ability to trigger apoptosis in premalignant or malignant cells *in vitro*, via the permeabilization of the outer mitochondrial membrane (32).

Sensitization of HT-29 Colon Cancer Cells to TRAIL-Induced Apoptosis by Quercetin Involves Death Receptor Congregation into Lipid Rafts

Pretreatment of HT-29 cells with quercetin enhanced the formation of the DISC in response to TRAIL stimulation. However, this effect was strong only for DR4 and caspase-8. Although in the current study, the expression of the death receptors was not modified by quercetin, it was shown that the polyphenol induced a clustered distribution of death receptors into raft domains of the plasma membrane in contrast to the homogenous distribution of the death receptors observed in untreated cells. DR4 and DR5 together with FADD and procaspase-8 were distributed in the fractions enriched in cholesterol and sphingolipids. These clusters could be caveolae, which have been reported previously to contain other death receptors, including Fas (18).

Lipid rafts play an important role in clustering or aggregating surface receptors, signaling enzymes, and adapter molecules into membrane complexes at specific sites and are essential for initiating signaling from several receptors. Several models for signal initiation in rafts have been proposed (33, 34). This redistribution induced by quercetin was not sufficient to trigger cell death, but it contributed to the sensitization to apoptosis induced by TRAIL. How this redistribution of the death receptors into lipid rafts occurs on treatment with quercetin remains a matter of speculation. We could hypothesize that quercetin, traversing through the cell membrane, induces hydrophobic modifications of the cell membrane lipids and by this way results in glycosphingolipid and cholesterol aggregation in the membrane. Previous studies have shown that quercetin induces ceramide aggregation and, thus, mitochondrial permeability transition, leading to apoptosis activation (35). In addition, it has been proposed that ceramide promotes TRAIL-induced apoptosis and that TRAIL-resistant cancers may benefit from combination therapy with ceramide or agents that enhance ceramide accumulation (36). Cyclooxygenase inhibitors can sensitize human colon cancer cells to TRAIL by inducing clustering of the DISC components into caveolae-rich plasma membrane domains (37). All these membrane events are getting to be characterized.

The cholesterol-sequestering molecule nystatin inhibited the surface aggregation of death receptors induced by quercetin and partially restored cell viability reduced by the combination of quercetin with TRAIL. All these data suggest that lipid raft localization of death receptors may be required for optimal cytotoxicity of quercetin and/or TRAIL. They also confirm that death receptor clustering into caveolae is critical for transmembrane signaling and amplification of the death signal (38).

Natural food products have gained considerable attention as cancer chemopreventive agents. The synergism of quercetin and TRAIL in apoptosis induction resulted by the functional complementation of activated extrinsic (death receptor) and intrinsic (mitochondrial) pathways. The potential role of quercetin to overcome the resistance of tumor cells against TRAIL provides an intriguing proposal for combination therapy.

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