

Akt-mediated eminent expression of c-FLIP and Mcl-1 confers acquired resistance to TRAIL-induced cytotoxicity to lung cancer cells

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Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a potential anticancer agent due to its selectivity in killing transformed cells. However, TRAIL can also stimulate the proliferation and metastasis of TRAIL-resistant cancer cells. Thus, acquired TRAIL resistance during TRAIL therapy would shift the patient's treatment from beneficial to detrimental. In this study, we focused on the acquired TRAIL resistance mechanism and showed that the elevated expression of the antiapoptotic factor cellular FLICE-like inhibitory protein (c-FLIP) and the prosurvival Bcl-2 family member myeloid cell leukemia-1 (Mcl-1) underlie the main mechanism of this type of TRAIL resistance in lung cancer cells. Chronic exposure to TRAIL resulted in lung cancer cell resistance to TRAIL-induced cytotoxicity, and this resistance was associated with the increase in the cellular levels of c-FLIP_L and Mcl-1_L. Overexpression of c-FLIP_L suppressed recruitment of caspase-8 to the death-inducing signaling complex, whereas increased Mcl-1_L expression blunted the mitochondrial apoptosis pathway. The elevation of c-FLIP_L and Mcl-1_L expression was due to Akt-mediated stabilization of these proteins in TRAIL-resistant cells. Importantly, suppressing c-FLIP_L and Mcl-1_L expression by RNA interference collectively alleviated acquired TRAIL resistance. Taken together, these results identify c-FLIP_L and Mcl-1_L as the major

determinants of acquired TRAIL resistance and could be molecular targets for improving the therapeutic value of TRAIL against lung cancer. [Mol Cancer Ther 2008;7(5):1156–63]

Introduction

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is regarded as the most promising anticancer agent in the TNF superfamily of cytokines because of its selective cytotoxicity in transformed cells (1, 2). However, numerous cancer cells are insensitive to TRAIL-induced cytotoxicity. The mechanism of this type of primary TRAIL resistance in cancer cells has been attributed to dysfunction of different steps in the TRAIL-induced apoptosis pathway and/or elevation of survival signals (3). In addition, cancer cells acquire TRAIL resistance during the course of TRAIL exposure; the mechanism of which is controversial (1, 4–7). Notably, TRAIL can promote proliferation and metastasis in TRAIL-resistant cancer cells (8). The acquired TRAIL resistance is particularly important for the clinical application of TRAIL because it may switch the effect of TRAIL from being beneficial to detrimental during the course of TRAIL treatment. Therefore, it is crucial to understand the mechanism of acquired TRAIL resistance to retain the cancer-killing activity of TRAIL while circumventing its cancer-promoting potential.

The cellular effects of TRAIL are mediated by its functional receptors, death receptor 4 (DR4)/TRAIL-R1 and death receptor 5 (DR5)/TRAIL-R2. Similar to TNF receptor 1, DR4 and DR5 are death receptors that contain a death domain in their cytoplasmic region to transduce cell death signals (1). They are also able to transmit signals leading to activation of the transcription factor nuclear factor- κ B and the c-Jun NH₂-terminal kinase (9, 10). Decoy receptor 1 (DcR1)/TRAIL-R3, decoy receptor 2 (DcR2)/TRAIL-R4, and osteoprotegerin can also bind TRAIL. Lacking a cytoplasmic region, DcR1 does not transmit signals into the cell. DcR2 has a truncated death domain, making it incapable of transmitting death signals while retaining the ability to mediate nuclear factor- κ B and c-Jun NH₂-terminal kinase activation (1). Both DcR1 and DcR2 function as decoy receptors to block TRAIL-induced apoptosis (1). Osteoprotegerin is a secreted TRAIL receptor that can also inhibit the therapeutic activity of TRAIL (11).

TRAIL-induced proapoptotic signals are transduced by the death-inducing signaling complex (DISC) that consists of the TRAIL receptors, TRADD, RIP, TRAF2, and FADD (1). RIP-dependent nuclear factor- κ B activation induced by TRAIL is antiapoptotic (9). Whether the role of TRAIL-induced c-Jun NH₂-terminal kinase activation is apoptotic

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or antiapoptotic is somewhat controversial and is believed to depend on the duration and extent of c-Jun NH₂-terminal kinase activation (12, 13). TRAIL-induced apoptosis is executed through FADD-mediated recruitment of caspase-8 and subsequent activation of downstream effector caspase-3 and caspase-7 (14). In addition, the apoptotic signal is further amplified through caspase-8-mediated cleavages of BID, a BH3-only member of the Bcl-2 family. The cleavage product, tBID, migrates to mitochondria and activates the mitochondrial apoptosis pathway. This process causes release of cytochrome *c* and Smac from mitochondria to the cytosol to induce caspase-9-mediated activation of effector caspases (14).

Several proteins act as negative regulators of TRAIL-induced apoptosis. The cellular FLICE-like inhibitory protein (c-FLIP), which is structurally similar to caspase-8, can be recruited to the DISC to inhibit the recruitment and activation of caspase-8 (3). In addition, the ratio of proapoptotic (Bax, Bak, and Bok) and antiapoptotic (Bcl-2 and Bcl-x_L) Bcl-2 family members can determine the cell's death or survival. Particularly, myeloid cell leukemia-1 (Mcl-1) recently has been attributed to TRAIL resistance in cancer cells (15, 16). Furthermore, the inhibitor of apoptosis proteins (IAP), including cIAP-1, cIAP-2, XIAP, and survivin, negatively regulate TRAIL-induced apoptosis through inhibiting caspases (14).

The purpose of this study was to elucidate the mechanism of acquired TRAIL resistance in lung cancer cells. We found that the expression of c-FLIP_L and Mcl-1_L is increased in an Akt-dependent manner in cells with acquired TRAIL resistance. Knockdown of c-FLIP_L and Mcl-1_L collectively alleviated TRAIL resistance. These results suggest that c-FLIP_L and Mcl-1_L play a key role in acquired TRAIL resistance, making them promising molecular targets for sensitizing TRAIL-induced lung cancer cell death.

Materials and Methods

Reagents

Glutathione *S*-transferase (GST)-TRAIL was prepared as described previously (9, 17). Human TNF was purchased from R&D Systems. MG132 and LY294002 were from Calbiochem. Adriamycin, etoposide, and cycloheximide were from Sigma. Antibodies against cIAP-1, cIAP-2, Bax, Bid, Akt, survivin, Bcl-2, caspase-3, and caspase-8 were from Santa Cruz Biotechnology. Anti-FADD, anti-DR4, anti-DR5, anti-DcR1, anti-DcR2, and anti-poly(ADP-ribose) polymerase were from BD Biosciences. Anti-β-actin was purchased from Sigma. Antibodies for Bcl-x_L, XIAP, BAD, cytochrome *c*, caspase-9, Akt, and phospho-Akt (Ser⁴⁷³) were from Cell Signaling. Antibodies against c-FLIP and Mcl-1 were from Alexis and Biovision, respectively.

Cell Culture and Induction of TRAIL-Resistant Cells

H460, H1568, and A549 cells were obtained from American Type Culture Collection and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mmol/L glutamate, 100 units/mL penicillin, and 100 μg/mL

streptomycin. To exclude the possibility that a population of TRAIL-resistant cells were selected during the course of establishment of TRAIL resistance, we cloned the cells and obtained five TRAIL-sensitive clones (which could be completely killed by treatment with 80 ng/mL TRAIL for 3 days) and pooled them for induction of TRAIL resistance. To induce TRAIL resistance, the TRAIL-sensitive H460 cells (designated as H460) were treated initially with a non-apoptotic dose (0.125 ng/mL) of TRAIL for 3 days and then split with fresh medium, with the TRAIL dose doubling every 3 days to a final dose of 128 ng/mL. The resulting cells were designated as H460 TRAIL-resistant (H460-TR) cells and maintained in medium containing 50 ng/mL TRAIL. For A549 and H1568 cells, the starting and final concentrations of TRAIL were 1 and 512 ng/mL, respectively. These TRAIL-resistant cells were designated as A549-TR and H1568-TR.

Cytotoxicity Assay

A cytotoxicity assay based on the release of lactate dehydrogenase was conducted using a cytotoxicity detection kit (Roche). Cells were seeded in 24-well plates at 70% to 80% confluence. After overnight culture, cells were treated as indicated in each figure legend. Lactate dehydrogenase release was determined as described previously (18, 19).

Western Blotting

Cell lysates were prepared by suspending cells in M2 buffer [20 mmol/L Tris-HCl (pH 7.6), 0.5% NP-40, 250 mmol/L NaCl, 3 mmol/L EDTA, 3 mmol/L EGTA, 2 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, 1 μg/mL leupeptin]. Equal amounts of protein from each cell lysate were resolved by 10%, 12%, or 15% SDS-PAGE and analyzed by Western blot. Cytosol was prepared as described (20). Cell fractions were prepared as described previously to detect cytochrome *c* release from mitochondria (20). The proteins were visualized by enhanced chemiluminescence following the manufacturer's instruction (Amersham). Each experiment was repeated at least thrice and representative results are shown. NIH image 1.62 was employed to quantify protein expression levels.

GST Pull-Down and DISC Analysis

Cells (1×10^8) were treated with 1 μg/mL GST-TRAIL for 20 min or left untreated. Cells were then washed twice with ice-cold PBS and lysed with 1 mL M2 buffer. The soluble fraction was incubated with 30 μL of 50% glutathione-Sepharose beads (Pharmacia) for 3 h on a rotator at 4°C. GST-TRAIL (1 μg/mL) was added to the lysates prepared from nonstimulated cells to precipitate the nonstimulated receptors. After five washes with M2 buffer, the bound proteins were eluted by boiling for 3 min in SDS-PAGE loading buffer and resolved in 10% SDS-PAGE. The presence of DR4, DR5, FADD, caspase-8, and c-FLIP was then determined by Western blot.

Knockdown of c-FLIP, Mcl-1, and Akt by RNA Interference

Short-interfering RNA (siRNA) for c-FLIP and Mcl-1 and the nontargeting siRNA (Silencer negative control 1 siRNA)

were obtained from Ambion. The two siRNAs had the following sense strand sequences: c-FLIP, GGGACCUU-CUGGAUAAUUUtt and Mcl-1, GGACUUUUUAUACCU-GUUAUtt. siRNAs against Akt1, Akt2, and Akt3 (siGENOME SMARTpool) were purchased from Dharmacon. H460-TR cells were seeded in a 12-well plate the day before transfection at ~50% confluency. siRNA was transfected with siRNA INTERFERin siRNA transfection reagent (Polyplus transfection). Thirty hours after transfection, the Mcl-1 and c-FLIP protein levels were measured by Western blot. TRAIL (80 ng/mL) was added to the culture 30 h (for Mcl-1 and c-FLIP siRNA) or 48 h (for Akt siRNA) post-transfection and incubated for 24 h to examine TRAIL-induced cytotoxicity.

Results

Establishment of Acquired TRAIL Resistance in H460 Cells

The human lung cancer-derived cell line H460 was used as a model to investigate the mechanism of acquired TRAIL resistance because these cells are relatively susceptible to TRAIL-induced cytotoxicity (Fig. 1A). H460 cells were initially treated with a nontoxic low dose (0.125 ng/mL) of TRAIL followed by exposure to 2-fold increased TRAIL concentrations every 3 days to a final dose of 128 ng/mL. The experiment was conducted with TRAIL-sensitive cells

(see Materials and Methods) and there was little cell death during the course of treatment; therefore, it is likely that TRAIL resistance is acquired rather than due to selection of a subpopulation of TRAIL-resistant cells. The H460-TR cells were remarkably insensitive to TRAIL-induced death (Fig. 1A), suggesting that acquired TRAIL resistance in H460 cells has been established by chronic exposure to nontoxic doses of TRAIL.

TRAIL-Induced Caspase-8 Activation Is Impaired in H460-TR Cells

To address the mechanism of acquired TRAIL resistance, we first examined caspase activation in H460-TR cells. Because caspase-8 is the initiator caspase that is essential for TRAIL-induced apoptosis, we compared activation of this key caspase between H460-TR and H460 cells. TRAIL treatment caused marked activation of caspase-8 activation in H460 cells as detected by cleavage of this caspase starting at 2 h post-treatment (Fig. 1B; data not shown). In contrast, activation of caspase-8 was significantly impaired in H460-TR cells (Fig. 1B). The activation of caspase-3, as well as cleavage of Bid and poly(ADP-ribose) polymerase by caspase-8 and caspase-3, respectively, was also suppressed in H460-TR cells (Fig. 1B). These results suggest that acquired TRAIL resistance in H460 cells may be, at least in part, due to suppression of caspase-8 activation, which occurs at an early stage of TRAIL receptor-mediated apoptosis signaling.

Increased c-FLIP Expression and Decreased Recruitment of Caspase-8 to DISC in H460-TR Cells

Because caspase-8 is recruited to and activated at the TRAIL-DISC during initiation of apoptosis, we next examined whether there is a defect in expression of the TRAIL-DISC components (1). There were no detectable changes in expression levels of TRAIL receptors (DR4, DR5, DcR1, and DcR2), TRADD, FADD, caspase-8, RIP, or TRAF2 in H460-TR cells. However, the expression of c-FLIP_L and c-FLIP_S was significantly increased in H460-TR cells (Fig. 2A). Because c-FLIP suppresses death receptor-mediated apoptosis by inhibiting the recruitment to and activation of caspase-8 at the DISC (3, 21), we examined DISC formation in H460-TR cells by a GST pull-down assay (see Materials and Methods). Pull-down of both DR4 and DR5 with GST-TRAIL was comparable between H460 and H460-TR cells (Fig. 2B; data not shown), suggesting that the exposure of these receptors on the cytoplasm membrane and binding to TRAIL were normally retained in H460-TR cells. Similarly, the recruitment of FADD to the DISC was comparable in H460 and H460-TR cells (Fig. 2B). In contrast, caspase-8 recruitment to the DISC was dramatically reduced in H460-TR cells. This reduction was accompanied by a dramatic increase in the recruitment of the modified form of c-FLIP_L (p43) to the DISC (Fig. 2B). It should be noted that after TRAIL treatment the c-FLIP_L protein was modified and became a 43-kDa band (p43) as detected by Western blot, which is consistent with previous reports (22, 23). The recruitment of c-FLIP_S was unchanged in H460-TR cells (Fig. 2B). These results suggest that increased expression and recruitment

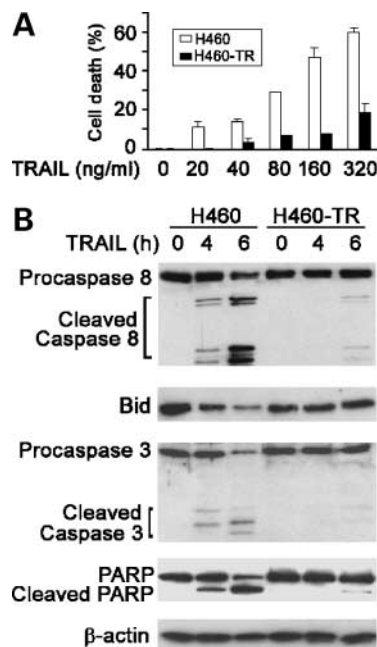
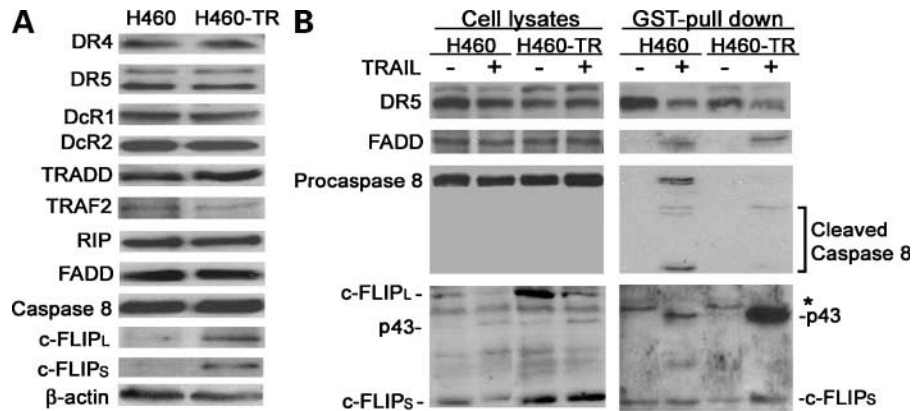


Figure 1. Chronic TRAIL exposure induces acquired resistance to TRAIL-induced cytotoxicity in H460 lung cancer cells. **A**, H460 and H460-TR cells were treated with indicated concentrations of TRAIL for 24 h. Cell death was measured by an LDH release assay. Columns, mean of three experiments; bars, SD. **B**, H460 and H460-TR cells were treated with 80 ng/mL TRAIL for the indicated times. Caspase-8, Bid, caspase-3, and PARP were detected by Western blot with specific antibodies against each of these proteins. β -actin was detected as an input control.

Figure 2. Increased c-FLIP_L expression and suppressed recruitment of caspase-8 to DISC in H460-TR cells. **A**, the expression of DISC components in H460 and H460-TR cells was detected by Western blot with specific antibodies against each protein. β -actin was detected as an input control. **B**, the DISC formation detected by GSTpull-down assay. H460 and H460-TR cells were treated with 1 μ g/mL GST-TRAIL for 20 min or left untreated. The cells were lysed in M2 buffer. GST-TRAIL (1 μ g/mL) was added to lysates from unstimulated cells to precipitate unstimulated TRAIL receptors. GST-TRAIL-bound protein complexes were analyzed for the presence of DR5, FADD, caspase-8, and c-FLIP by Western blot. *, non-specific band.



of c-FLIP_L, but not c-FLIP_S, to the DISC contributes to blocking caspase-8 activation in H460-TR cells. Therefore, we focused our studies on c-FLIP_L.

c-FLIP expression is regulated at the transcriptional and post-translational levels (24). The increase of c-FLIP_L protein in H460-TR cells is unlikely through higher transcription rates because no significant difference in mRNA expression of c-FLIP_L was observed between H460 and H460-TR cells (data not shown). However, when protein synthesis was blocked by cycloheximide, c-FLIP_L protein rapidly turned over in H460-TR cells; this turnover could be reversed by the proteasome inhibitor MG132, suggesting that proteasomal degradation is pivotal in regulating c-FLIP_L expression in these cells (Fig. 3A). Therefore, we compared the stability of c-FLIP_L in H460 and H460-TR cells by shutting off protein synthesis with cycloheximide and chasing c-FLIP_L expression for up to 4 h. The half-life of c-FLIP_L in H460-TR cells was \sim 180 min, much longer than that of the H460 cells (\sim 75 min; Fig. 3B and C). These results imply that increased protein stability in H460-TR cells contributes to increases in c-FLIP_L protein levels, which leads to the suppression of caspase-8 recruitment and activation during TRAIL-induced apoptotic signaling in H460-TR cells.

Elevated Expression of Mcl-1_L and Impaired Mitochondrial Apoptosis Pathway in H460-TR cells

Several proteins, such as members of the IAP and Bcl-2 families, also affect TRAIL-induced cell death (3). Therefore, we examined the expression levels of these proteins by Western blot analysis. As shown in Fig. 4A, no significant changes in expression levels of IAP family members, including survivin, XIAP, cIAP1, and cIAP2, were observed in H460-TR cells. Among the Bcl-2 family proteins examined, only the expression level of Mcl-1_L was dramatically increased. Interestingly, the expression of Mcl-1_S remained unchanged (Fig. 4A). Although minor changes of Bcl-x_L and BAD were seen occasionally, this observation was inconstant; we thus focused on Mcl-1_L. Because Mcl-1_L negatively regulates the mitochondria-mediated apoptosis pathway, we examined whether this pathway is blocked in H460-TR cells. Indeed, TRAIL-induced release of cytochrome *c* from mitochondria and

activation of caspase-9 were greatly reduced in H460-TR cells (Fig. 4B). These results suggest that blocking the intrinsic apoptosis pathway by increased Mcl-1_L expression may underlie another mechanism of acquired TRAIL resistance in H460-TR cells.

We further investigated the mechanism of the Mcl-1_L increase in H460-TR cells and detected no alteration in expression of Mcl-1_L mRNA (data not shown). Proteasomal degradation appeared to be important in controlling the expression level of Mcl-1_L in H460-TR cells because MG132 effectively blocked the decrease of Mcl-1_L levels when protein synthesis was blocked (Fig. 4C). The Mcl-1_L protein

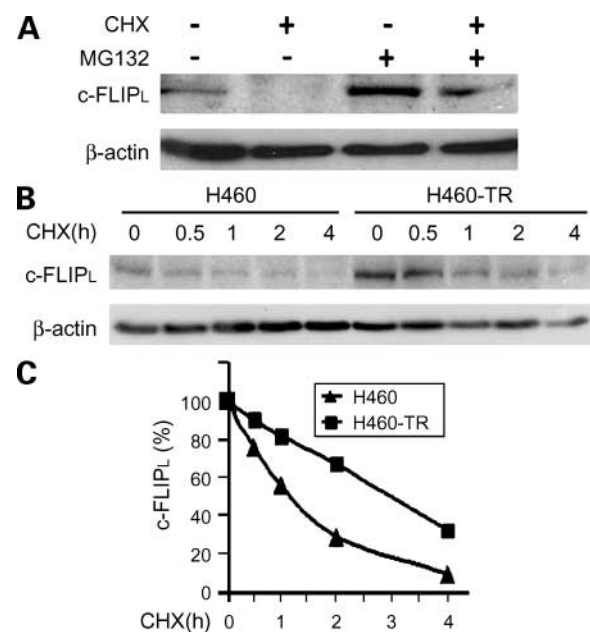


Figure 3. Increased protein stability of c-FLIP_L in H460-TR cells. **A**, H460-TR cells were treated with either 10 μ g/mL cycloheximide (CHX) or 10 μ M MG132 or both for 4 h. c-FLIP_L was detected by Western blot. β -actin was detected as an input control. **B**, H460 and H460-TR cells were treated with 10 μ g/mL CHX for indicated times. c-FLIP_L protein was detected by Western blot. β -actin was detected as an input control. **C**, the results of c-FLIP_L expression, shown in panel (B), were quantified and normalized to β -actin.

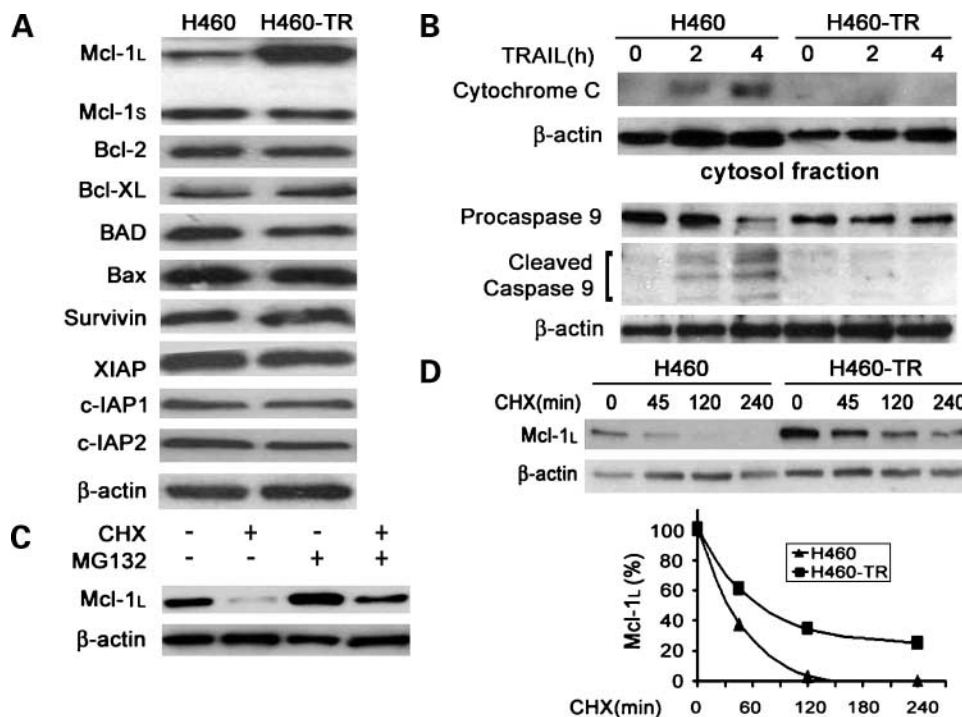


Figure 4. Increased Mcl-1_L expression and suppressed mitochondrial apoptosis pathway in H460-TR cells. **A**, expression of Bcl-2 family and IAP family proteins in H460 and H460-TR cells was detected by Western blot analysis. β -actin was detected as an input control. **B**, H460 and H460-TR cells were treated with 80 ng/mL TRAIL for the indicated times. The cytosolic fraction of cell extracts was probed for cytochrome C (upper panel). Total cell extracts from TRAIL-treated (80 ng/ml) H460 and H460-TR cells were detected for caspase-9 by Western blot. **C**, H460-TR cells were treated with either 10 μ M cycloheximide (CHX) or 10 μ M MG132 or both for 4 h. Mcl-1_L protein was detected by Western blot. β -actin was detected as an input control. **D**, H460 and H460-TR cells were treated with 10 μ M CHX for indicated times. Mcl-1_L protein was analyzed by Western blot. β -actin was detected as an input control (upper panel). The results of Mcl-1_L expression were quantified and normalized to β -actin (lower panel).

has a prolonged half-life (80 min) in H460-TR cells compared with that of the H460 cells (30 min; Fig. 4D). These results indicate that increased protein stability led to the up-regulation of Mcl-1_L, contributing to the suppression of mitochondria-mediated apoptosis in H460-TR cells.

Suppression of c-FLIP_L and Mcl-1_L Expression Concurrently Alleviates Acquired TRAIL Resistance

We further confirmed the role of c-FLIP_L and Mcl-1_L in acquired TRAIL resistance by reducing the expression of these proteins. Because both c-FLIP_L and Mcl-1_L are short-lived proteins that can be effectively suppressed by protein synthesis inhibitors (Figs. 3B and 4D), we assessed the effect of cycloheximide on TRAIL-induced cytotoxicity in H460-TR cells (Fig. 5A). Cycloheximide restored TRAIL-induced cytotoxicity, concomitant to a dramatic reduction of c-FLIP_L or Mcl-1_L expression in H460-TR cells. Additionally, knockdown of c-FLIP_L and Mcl-1_L expression was further used to verify the role of these proteins in acquired TRAIL resistance (Fig. 5B). Transfection of control siRNA had no detectable effect; however, suppressing expression of either c-FLIP_L or Mcl-1_L partially alleviated acquired TRAIL resistance (Fig. 5C). Importantly, concurrent knockdown of c-FLIP_L and Mcl-1_L cooperatively sensitized H460-TR cells to TRAIL-induced cytotoxicity (Fig. 5C), indicating that both c-FLIP_L and Mcl-1 are important in acquired TRAIL resistance.

Elevated Akt Activity Contributes to Stabilization of Mcl-1_L and c-FLIP_L

Akt is a master kinase involved in cell survival (25). Recently, it was reported that Akt positively regulates Mcl-1 and c-FLIP expression (26–28). Interestingly, Akt activity was markedly increased in H460-TR cells (Fig. 6A). To

investigate whether the elevated Akt activity contributes to the increased protein stability of Mcl-1_L and c-FLIP_L, H460-TR cells were treated with the specific PI3-kinase inhibitor LY294002 to suppress Akt activity. Blocking Akt by LY294002 reduced Mcl-1_L and c-FLIP_L expression and alleviated TRAIL resistance of H460-TR cells (Fig. 6B; data not shown). To further confirm the role of Akt in regulating Mcl-1_L and c-FLIP_L protein levels, RNA interference was used to knock down Akt expression by concurrently targeting all of its three isoforms, Akt1, Akt2, and Akt3, in H460-TR cells. Marked down-regulation of Mcl-1_L and c-FLIP_L was achieved by suppressing Akt expression and activity (Fig. 6C). Moreover, knockdown of Akt significantly restored sensitivity to TRAIL-induced cytotoxicity in H460-TR cells (Fig. 6D), indicating that Akt contributes substantially to acquired TRAIL resistance in lung cancer cells through regulating Mcl-1_L and c-FLIP_L expression.

Discussion

In this study, we showed that the acquired TRAIL resistance is associated with enhanced expression of c-FLIP_L and Mcl-1_L in H460 lung cancer cells. Modified c-FLIP_L was preferentially recruited to the TRAIL DISC and suppressed recruitment and activation of caspase-8, thereby inhibiting the extrinsic apoptosis pathway. The increased Mcl-1_L level contributes to suppressing the mitochondrial apoptosis pathway. Inhibition of FLIP_L and Mcl-1_L expression by RNA interference cooperatively alleviated the acquired TRAIL resistance. Thus, acquired resistance to TRAIL-induced cytotoxicity was due to suppressing both extrinsic and intrinsic apoptosis pathways.

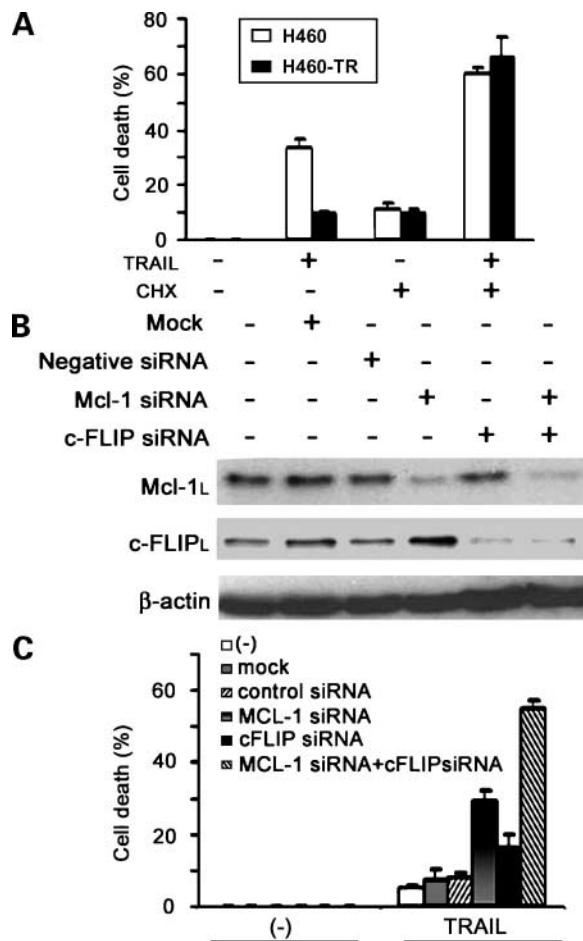


Figure 5. Suppressing c-FLIP_L and Mcl-1_L expression in H460-TR cells alleviates acquired TRAIL resistance. **A**, H460-TR cells were left untreated or treated with 10 μg/mL cycloheximide (CHX) for 30 min followed by treatment with 80 ng/mL TRAIL for 24 h. Cell death was measured by LDH assay. Columns, mean of three experiments; bars, SD. **B**, H460-TR cells were transfected with either Mcl-1 siRNA, c-FLIP siRNA, or both. Mcl-1_L and c-FLIP_L were assessed by Western blot 30 h after transfection. Nontransfected, mock, or negative siRNA-transfected cells also are shown as controls. **C**, H460-TR cells were transfected with indicated siRNA. Thirty hours after transfection, cells were treated with 80 ng/mL TRAIL for another 24 h and cell death was detected as described in (A).

We further showed that elevated Akt activity is crucial for overexpression of c-FLIP_L and Mcl-1_L. Similar observations were also made in H1568 and A549 cells (data not shown). Collectively, data from this study show that the Akt-mediated increase of c-FLIP_L and Mcl-1_L levels underlies the main mechanism of acquired TRAIL resistance in lung cancer. Therefore, Akt, c-FLIP_L, and Mcl-1_L could be useful targets for circumventing acquired TRAIL resistance in lung cancer cells.

As a structural homologue of caspase-8, c-FLIP binds FADD, competing with caspase-8 for recruitment to DISC. c-FLIP suppression is assumed to be involved in mediating the sensitization of cancer cells to TNF- or TRAIL-induced apoptosis by cycloheximide or the transcription inhibitor actinomycin D (29). In several types of cancers, increased

c-FLIP expression is correlated with resistance to TRAIL-, Fas-, or TNF-induced apoptosis (21, 23, 30–32). However, the mechanisms underlying the c-FLIP expression increase have not been well elucidated. Post-translational modification and degradation of c-FLIP as well as increased mRNA levels have been suggested to be involved in increasing c-FLIP expression (24). In this study, chronic TRAIL exposure induced c-FLIP_L expression in lung cancer cells likely through post-transcriptional regulation. Although both long and short forms of c-FLIP were increased, only c-FLIP_L was preferentially recruited to the TRAIL DISC. Because the recruited c-FLIP_L was the proteolyzed form (p43) and the extent of c-FLIP_L recruitment to the DISC was enhanced in relation to the protein expression levels (Fig. 3), it is plausible that an unidentified mechanism that modifies c-FLIP_L may have been activated during chronic TRAIL exposure to facilitate recruitment of c-FLIP_L to the DISC. Furthermore, it is noteworthy that although both c-FLIP_L and c-FLIP_S are apoptosis inhibitors, their contributions to apoptosis regulation, specifically to acquired TRAIL resistance, could be distinct. c-FLIP_L was reported to be more significant in the resistance of cancer cells to therapy (21, 30). Interestingly, increased c-FLIP_S did not block apoptosis induced by TRAIL plus the proteasome inhibitor bortezomib (33).

Increased expression of antiapoptotic Bcl-2 family members such as Bcl-x_L and Mcl-1 is associated with the resistance of cancer cells to therapy, suggesting that the intrinsic pathway also plays a role in resistance to therapeutics (34). Mcl-1 is involved in the resistance of lung cancer cells to apoptosis induced by chemotherapeutics, ionizing radiation, and TRAIL (35, 36). Mcl-1_L can block the tBID-mediated activation of the mitochondrial apoptosis pathway induced by TRAIL (15). Of the two Mcl-1 isoforms, Mcl-1_L is widely regarded as an antiapoptotic factor, whereas Mcl-1_S is found to be proapoptotic (37). Thus, an increase in the ratio of Mcl-1_L to Mcl-1_S would elevate the apoptosis threshold in cancer cells. Interestingly, we show that the expression of Mcl-1_L but not Mcl-1_S was dramatically increased after chronic exposure to TRAIL. The increased Mcl-1_L expression was correlated with inhibiting TRAIL-induced cytochrome *c* release from mitochondria and activation of caspase-9. Knockdown of Mcl-1_L partially alleviated TRAIL resistance, indicating that suppressing the mitochondrial apoptosis pathway by Mcl-1_L at least is partly involved in the mechanism of acquired TRAIL resistance.

We further found that increased Akt activity is accompanied by acquired TRAIL resistance in lung cancer cells. Suppressing Akt activity with the phosphatidylinositol 3-kinase inhibitor LY294002 or Akt siRNA suppressed expression of both c-FLIP_L and Mcl-1_L. This finding is consistent with other reports showing that Akt regulates these two survival factors in cancer cells (26, 38) and with our recent findings that inhibited Akt activity dramatically sensitizes lung cancer cells to TNF- or TRAIL-induced cytotoxicity (17, 39). Although our data suggest that Akt may enhance the expression of c-FLIP_L and Mcl-1_L by

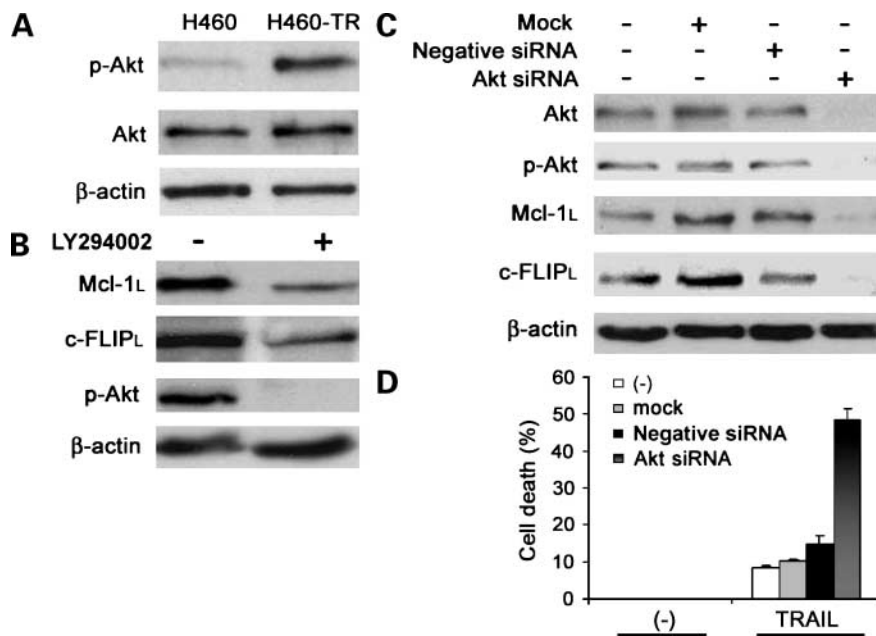


Figure 6. Elevated Akt activity contributes to stabilization of Mcl-1_L and c-FLIP_L. **A**, phosphorylated-Akt (p-Akt) and total Akt in H460 and H460-TR cells were measured by Western blot. β-actin was detected as an input control. **B**, H460-TR cells were treated with 10 μM LY294002 overnight. Mcl-1_L, c-FLIP_L, and p-Akt were detected by Western blot. β-actin was detected as an input control. **C**, pooled siRNA against Akt1, Akt2, and Akt3 was used to knock down Akt in H460-TR cells. p-Akt, total Akt, Mcl-1_L, and c-FLIP_L were measured by Western blot. **D**, non-transfected, mock, and negative siRNA-transfected cells are shown as in H460-TR cells. Control cells were treated with 80 ng/mL TRAIL for 24 h and cell death was detected by LDH assay. Columns, mean of three experiments; bars, SD.

affecting the protein degradation pathway, the exact mechanisms underlying such enhancement is not clear currently. As a glycogen synthase kinase 3 inhibitor, Akt may increase the stability of Mcl-1 by suppressing glycogen synthase kinase 3β. Phosphorylation of Mcl-1 by glycogen synthase kinase 3 recently has been shown to enhance Mcl-1 protein turnover (40). Akt activation also was associated with suppressing glycogen synthase kinase 3 and increasing the c-FLIP expression level (41). Whether the regulation of Mcl-1 and c-FLIP is activated through the same mechanism and whether it involves glycogen synthase kinase 3 remain to be elucidated.

Direct evidence showing that increased expression of c-FLIP_L and Mcl-1_L is the main determinant of acquired TRAIL resistance in lung cancer cells came from the siRNA knockdown experiment in which concurrent knockdown of these two proteins additively sensitized H460-TR cells to TRAIL-induced cytotoxicity. Therefore, c-FLIP_L and Mcl-1_L could be ideal molecular targets for attenuating acquired TRAIL resistance to lung cancer. It is remarkable that TRAIL can stimulate the proliferation and metastasis of TRAIL-resistant cancer cells (8). If it arises during therapy, such acquired TRAIL resistance would shift the TRAIL treatment from beneficial to detrimental. Thus, preventing or attenuating acquired TRAIL resistance by targeting c-FLIP_L and Mcl-1_L could be crucial in retaining the cancer-killing activity of TRAIL and circumventing its cancer-promoting potential. *In vivo* experiments with animal models are needed to verify the effect of this approach in sensitizing TRAIL for lung cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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