Upregulation of $FLIP_S$ by Akt, a possible inhibition mechanism of TRAIL-induced apoptosis in human gastric cancers

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent inducer of apoptosis in some, but not all cancer cells. To assess the regulation of TRAIL-resistance in the human gastric cancer cells, we examined TRAIL sensitivity, TRAIL receptor expression, and intracellular signaling events induced by TRAIL. All the gastric cancer cell lines tested were susceptible to TRAIL to some extent, except for SNU-216 cell line, which was completely resistant. TRAIL receptor expression was not related to the TRAIL-sensitivity. Of the cell lines tested, SNU-216 showed the highest level of constitutively active Akt and the short form of FLICE inhibitory protein ($FLIP_S$). Treatment with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 or with the protein synthesis inhibitor cycloheximide induced a suppression of constitutive Akt activation in SNU-216 cells and a concomitant decrease in the expression of $FLIP_S$. The reduction of Akt activity by LY294002 affected the transcriptional level of $FLIP_S$, but not the mRNA stability. As a result, LY294002 or cycloheximide significantly enhanced TRAIL-induced apoptosis. Moreover, the overexpression of constitutively active Akt in the TRAIL-sensitive cell line, SNU-668, rendered the cell line resistant to TRAIL. In addition, infection of the same cell line with retrovirus expressing $FLIP_S$ completely inhibited TRAIL-induced apoptosis by blocking the activation of caspase-8. Therefore, our results suggest that Akt activity promotes human gastric cancer cell survival against TRAIL-induced apoptosis via upregulation of $FLIP_S$, and that the cytotoxic effect of TRAIL can be enhanced by modulating the Akt/$FLIP_S$ pathway in human gastric cancers. (Cancer Sci 2003; 94: 1066–1073)

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the *tumor necrosis factor* (*TNF*) gene family, and shares amino acid homology with TNF and Fas ligand (FasL).[1,2] TRAIL is a potent inducer of apoptosis in a wide variety of tumor cells, but does not cause toxicity in the majority of normal cells.[3,4] Thus, TRAIL may be suitable for novel anti-cancer therapies.

The TRAIL apoptotic signal is transduced through the cell surface death receptors DR4/TRAIL-R1 and DR5/TRAIL-R2/TRICK-2. TRAIL also binds to decoy receptors, DcR1/TRAIL-R3/TRID/LIT and DcR2/TRAIL-R4/TRUNDD, which inhibit TRAIL signaling.[5–7] Recent studies have shown that TRAIL triggers apoptosis by recruiting the initiator procaspase-8 through the adaptor protein FADD.[8,9] Caspase-8 can directly activate downstream effector caspases, including the procaspase-3, -6, and -7.[10] In an alternative death signal pathway, caspase-8 cleaves Bid and triggers mitochondrial damage that in turn leads to cytochrome *c* release.[11,12] Cytochrome *c* in the cytoplasm then binds to Apaf-1, and activates caspase-9. Subsequently, caspase-9 activates caspase-3, which induces poly(ADP-ribose) polymerase (PARP) cleavage.[13]

Although TRAIL selectively induces apoptosis in many types of cancers, some tumors are resistant to TRAIL-induced apoptosis. The serine/threonine kinase Akt/protein kinase B (PKB) mediates cell survival signaling, which is initiated by various growth factors. Recent studies have demonstrated that elevated Akt activity upregulates FLIP and inhibits TRAIL-induced apoptosis in prostate and renal cancer cells.[14–17] In total, results available to date indicate that Akt may be a key death regulator in the TRAIL-induced apoptosis and that FLIP expression may be regulated by the PI3K/Akt pathway in some cancer cells.

FLIP is a cytoplasmic protein that has sequence homology with caspase-8 (FLICE) and is capable of inhibiting apoptosis by blocking the recruitment and processing of caspase-8.[18,19] Several spliced variants of FLIP, including the full-length long form ($FLIP_L$) and the short form ($FLIP_S$), have been identified in mammalian cells. However, the association of $FLIP_L$ or $FLIP_S$ expression with TRAIL-induced apoptosis varies in different cancer types. In leukemic progenitor cells, both $FLIP_L$ and $FLIP_S$ interact with caspase-8 and inhibit TRAIL-mediated apoptosis.[20] A high level of $FLIP_L$ correlated with resistance to TRAIL-induced apoptosis in bladder carcinoma cells.[21] In comparison, it has been reported that the $FLIP_S$ level was positively correlated with TRAIL resistance in prostate cancer and head and neck cancer.[17,22] Other studies using colon cancer, some melanomas and Burkitt's lymphoma failed to identify a link between FLIP expression and TRAIL resistance.[23–25] However, until now, the different roles and the distinct regulation of $FLIP_S$ and $FLIP_L$ in the TRAIL-induced apoptosis in gastric cancer have not been reported.

Gastric cancer is one of the most common malignancies worldwide and the major cause of cancer death in Asian countries.[26] However, the cure rate of this disease is limited because of the ineffectiveness of chemotherapy and radiotherapy. Thus, we need to develop new treatment modalities for gastric carcinoma. In the present study, we compared the TRAIL-sensitivities of different gastric cancer cell lines. In order to investigate the molecular mechanisms that might contribute to TRAIL-resistance of gastric cancer cells, we examined the role of the Akt/FLIP pathway in the TRAIL-signaling cascade of human gastric cancer and the differential regulation of splicing variants of FLIP by Akt activity.

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The abbreviations used are: DcR1, decoy receptor 1; DcR2, decoy receptor 2; DR4, death receptor 4; DR5, death receptor 5; EGFP, enhanced green fluorescent protein; FADD, Fas-associated death domain; FasL, Fas ligand; FLICE, Fas-associated death domain-like interleukin-1β-converting enzyme; FLIP, FLICE inhibitory protein; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; PI3K, phosphatidylinositol-3 kinase; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Materials and Methods

Reagents and antibodies. Polyclonal anti-caspase-3 antibody was purchased from Santa Cruz (Santa Cruz, CA), anti-DcR2 and anti-DR5 from Stressgen (Victoria, BC, Canada), anti-DR4 from Imgenex (San Diego, CA), anti-DcR1 from R&D Systems, Inc. (Minneapolis, MN), anti-$FLIP_L$, anti-$FLIP_S$ and anti-caspase-9 from Calbiochem (Darmstadt, Germany), and anti-Bid, anti-phospho-Akt and anti-Akt from New England Biolabs (Beverly, MA). Monoclonal antibodies were purchased from the following companies; anti-caspase-8 from Upstate Biotechnology (Lake Placid, NY), anti-FADD from Stressgen, and anti-PARP from Biomol Research Laboratory (Plymouth Meeting, PA). Other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture and assessment of cell viability. The human gastric cancer cell lines, SNU-1, SNU-5, SNU-216, SNU-601, SNU-668, and SNU-719, were obtained from the Korean Cell Line Bank (Seoul, Korea). All cell lines were cultured in RPMI medium with 10% fetal bovine serum. The cultures were maintained in a 37°C humidified incubator in a mixture of 95% air and 5% CO_2. To determine cell viability, gastric cancer cell lines were seeded into 96-well plates at a density of 1×10^4 cells per well one day prior to the experiment. Cells were treated with TRAIL (200 ng/ml)[27] in the presence or absence of cycloheximide (1 μg/ml) for 24 h. To measure the viability of adherent cells, such as SNU-216, SNU-601, SNU-668, and SNU-719, the wells were stained with 0.2% crystal violet and absorbance was measured at 570 nm using an ELISA reader (Bio-Rad, Hercules, CA) as described previously.[28] The viability of the suspension cell lines, SNU-1 and SNU-5, was measured by trypan blue exclusion assay.[29] In the case of the dose-dependency study, SNU-216 and SNU-668 cells were treated with increasing concentrations of TRAIL (0, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 150, and 200 ng/ml) with or without cycloheximide (1 μg/ml).

Morphological evaluation. Approximately 5×10^5 cells were plated into 60-mm dishes and incubated overnight. Cells were pretreated either with cycloheximide (1 μg/ml) or with LY294002 (50 μ*M*) for 1 h before being treated with TRAIL (100 ng/ml) for 24 h and then analyzed by phase-contrast microscopy for signs of apoptosis.

Polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis. Cells were lysed with 1× Laemmli lysis buffer [2.4 *M* glycerol, 0.14 *M* Tris (pH 6.8), 0.21 *M* sodium dodecyl sulfate (SDS), 0.3 m*M* bromophenol blue] and then boiled for 10 min. Protein content was measured using BCA Protein Assay Reagent (Pierce, Rockford, IL). Samples were diluted with 1× lysis buffer containing 1.28 *M* β-mercaptoethanol, and equal amounts of protein were loaded onto 8–10% SDS-polyacrylamide gels. Proteins were electrophoretically transferred to a nitrocellulose membrane, which was blocked with 7.5% nonfat dry milk in PBS-Tween-20 (0.1%, v/v) at 4°C overnight. The membrane was incubated with either anti-PARP, anti-caspase-8, anti-Bid, anti-caspase-9, anti-caspase-3, anti-DcR1, anti-DcR2, anti-DR4, anti-DR5, anti-$FLIP_L$, anti-$FLIP_S$, anti-FADD, anti-phospho-Akt, or anti-Akt antibody (diluted according to the manufacturer's instructions) for 3 h. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody for 1 h. Immunoreactive protein was visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Immunoprecipitation and Akt kinase assay. Cells were lysed in 1 ml of ice-cold lysis buffer containing 20 m*M* Tris (pH 7.5), 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1% Triton X-100, 2.5 m*M* sodium pyrophosphate, 1 m*M* β-glycerol phosphate, 1 m*M* Na_3VO_4, 1 μg/ml leupeptin, and 1 m*M* PMSF. After centrifugation, the supernatants were incubated with agarose beads crosslinked with anti-Akt antibody at 4°C overnight. Immunoprecipitates were washed twice with the lysis buffer and twice with kinase buffer [25 m*M* Tris (pH 7.5), 5 m*M* β-glycerol phosphate, 2 m*M* DTT, 0.1 mM Na_3VO_4, and 10 m*M* $MgCl_2$]. Akt kinase activity was measured using an Akt kinase assay kit (Cell Signaling Technology, Inc., Beverly, MA) with GSK-3 fusion protein as a substrate according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction. Total RNA was extracted using TRIzol (Gibco BRL Life Technologies, Grand Island, NY) following the manufacturer's instructions, and converted to cDNA using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega, Madison, WI) with oligo(dT) primers (Novagen, Milwaukee, WI). To detect $FLIP_L$ and $FLIP_S$ mRNA, 5 μl of the resultant cDNA was added to 50 μl of PCR mixture containing 1× PCR buffer, 2.5 units of *Taq* DNA polymerase, 1.5 m*M* $MgCl_2$, 200 μ*M* dNTPs and 20 pmol of each specific primer. The following specific primers were used: $FLIP_L$, 5′-AATTCAAGGCTCAGAAGCGA-3′ and 5′-GGCAGAAACTCTGCTGTTCC-3′; $FLIP_S$, 5′-ATGTCTGCTGAAGTCATCCAT-3′ and 5′-TCACATGGAACAATTTCCAAG-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TGCCGTCTAGAAAAACCTGC-3′ and 5′-ACCCTGTTGCTGTAGCCAAA-3′. The PCR cycling conditions were as follows: 1 cycle at 94°C for 2 min and then 35 cycles at 94°C for 40 s, 60°C for 40 s and 72°C for 40 s, and a final extension for 10 min at 72°C. Products were separated in 1.5% agarose gel containing 0.5 μg/ml of ethidium bromide. For quantitative RT-PCR, primers were chosen by using Primer Express 2.0 (Applied Biosystems, Foster City, CA). The following specific primers were used: $FLIP_L$, 5′-GAGGCTCCCAGAGTGTGTATGG-3′ and 5′-TGGCCCTCTGACACCACATAG-3′; $FLIP_S$, 5′-AATGTTCTCCAAGCAGCAATCC-3′ and 5′-CCAAGAATTTTCAGATCAGGACAAT-3′. PCR amplification with SYBR Green PCR Master Mix was performed by using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The relative abundance of products, normalized with respect to GAPDH, was calculated from threshold cycle (C_T) numbers, ΔC_T (target C_T−GAPDH C_T), according to the manufacturer's paradigm.

Transfection. In order to generate constitutively active Akt (CA-Akt)-overexpressing SNU-668 cells, cells were transiently transfected with pUSEamp-CA-Akt (Upstate Biotechnology) or pUSEamp (empty vector control) using Lipofectamine Plus (Gibco BRL Life Technologies). Expression levels of CA-Akt were determined by immunoblot analysis.

Retroviral vector construction and virus infection. The retroviral vector MFG.EGFP was described previously.[30] The retroviral vector containing $FLIP_S$/EGFP (MFG.$FLIP_S$/EGFP) was constructed by replacing the EGFP (enhanced green fluorescence protein) coding sequence of MFG.EGFP with the $FLIP_S$/EGFP coding sequence obtained by PCR amplification of pEGFP-N1.$FLIP_S$ (kindly provided by Dr. H. Wajant). The detailed strategy of cloning is available upon request. To generate a retrovirus-producing cell line, the retroviral vector, plasmids were introduced into the PA317 packaging cell line by the calcium phosphate precipitation method.[31] Two days later, drug-resistant cells were selected in a selective medium containing 2 μg/ml of puromycin (Sigma). The viral supernatants were harvested from overnight cultures of the mixed population of drug-resistant PA317 cells at semi-confluency. The viral infection was performed by incubating SNU-668 cells with the viral supernatant for 2 h in the presence of 4 μg/ml Polybrene (Sigma). Forty-eight hours after the infection, the cells were placed in a selective medium containing 2 μg/ml puromycin. After 10 days of selection, the drug-resistant cells were harvested and stored for further analysis.

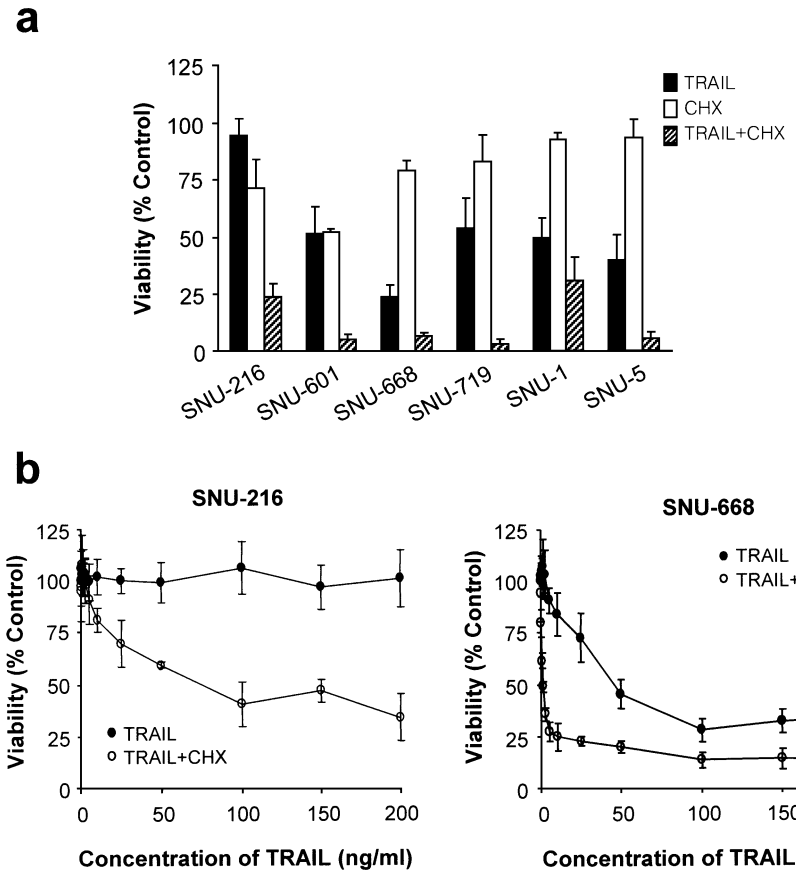

Fig. 1. Cytotoxic effects of TRAIL on human gastric cancer cell lines. (a) Cells were treated with 200 ng/ml of TRAIL with or without cycloheximide (CHX) (1 μg/ml) for 24 h. Cell survivals represent the mean percentage survivals compare to untreated cells and bars represent SD (n=4). (b) Effects of various doses of TRAIL on the viability of SNU-216 and SNU-668 cell lines. Cells were treated with various concentrations (0.05–200 ng/ml) of TRAIL with or without CHX (1 μg/ml) for 24 h. Values represent the means±SD of triplicate samples in three experiments.

Results

Cytotoxic effects of TRAIL on human gastric cancer cell lines. To assess the cytotoxicity of TRAIL, six human gastric cancer cell lines were treated with 200 ng/ml of TRAIL in the presence or absence of cycloheximide (1 μg/ml) for 24 h. Cell viability assay demonstrated that SNU-668 was highly sensitive to TRAIL-induced cell death, and that SNU-601, SNU-719, SNU-1 and SNU-5 were moderately sensitive, whereas SNU-216 was almost completely resistant (Fig. 1a, b). However, combined treatment with TRAIL (200 ng/ml) and cycloheximide (1 μg/ml) for 24 h increased cell death in all cell lines.

SNU-216, a TRAIL-resistant cell line, and SNU-668, a TRAIL-sensitive cell line, were further tested to determine the dose dependency of TRAIL-induced cell death (Fig. 1b). TRAIL alone reduced the survival of SNU-668 cells at doses of 0.25–200 ng/ml in a dose-dependent manner, whereas SNU-216 proved to be resistant to TRAIL in the same concentration range. The combination of TRAIL and cycloheximide (1 μg/ml) increased cell death in both SNU-216 and SNU-668 cell lines.

Expression of TRAIL receptors and FADD in human gastric cancer cell lines. To identify whether expression of TRAIL receptors is linked with TRAIL-sensitivity in gastric cancer cells, we determined the expression levels of DR4, DR5, DcR1, and DcR2 by western blotting (Fig. 2). All human gastric cancer cell lines expressed DR4, DR5, and DcR2, although at variable levels. On the other hand, DcR1 was expressed in only SNU-216 and SNU-668, at very low levels. Thus, these findings indicate that TRAIL-sensitivity in gastric cancer cell lines is primarily regulated at the intracellular level, rather than at the receptor level.

In addition, we examined the expression of the adapter protein FADD to determine whether the loss of FADD is correlated with TRAIL-resistance, and found that the adapter protein FADD was expressed in all cell lines tested, but the levels were not correlated with TRAIL-sensitivity (Fig. 2).

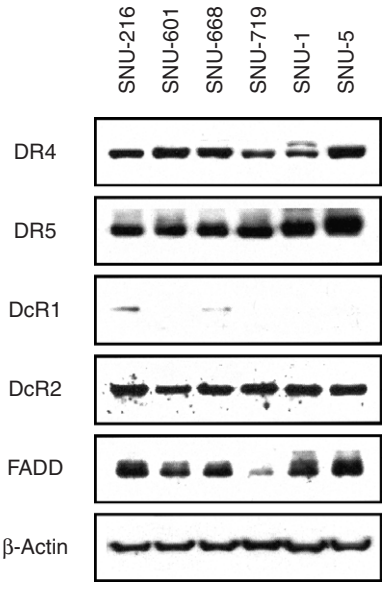

Fig. 2. Expressions of TRAIL receptors and FADD in human gastric cancer cell lines. The cell lines indicated were harvested and the lysates from equal amounts of protein (20 μg) were separated by SDS-PAGE. Membranes were immunoblotted using primary antibodies against DR4, DR5, DcR1, DcR2, or FADD. β-Actin was used as the internal standard.

Akt activation and $FLIP_S$ expression in gastric cancer cell lines. In order to examine the relationship between Akt and TRAIL-resistance in gastric cancer cells, we examined the activity of Akt by kinase assay (Fig. 3a). Of the six gastric cancer cell lines tested, the highest activity of Akt was found in the TRAIL-resistant cell line SNU-216, whereas moderate activities of Akt in SNU-601 and SNU-719, and lower activities in SNU-668, SNU-1 and SNU-5 were found. These results suggest that the activation of Akt is probably related with TRAIL-resistance.

Since the association of FLIP expression with TRAIL-induced apoptosis varies in different cancer types, we further examined the expression levels of FLIP to determine whether there is any correlation between FLIP and TRAIL-sensitivity in gastric cancer cells. FLIP expression was detected by immunoblot using antibodies that recognize both $FLIP_L$ and $FLIP_S$, the splice variants of FLIP (Fig. 3b). $FLIP_L$ expression was detected in all tested cell lines, but did not show any significant difference among cell lines. In contrast, $FLIP_S$ was very highly expressed in only SNU-216. Moreover, the expression of $FLIP_S$ appears to correlate with the constitutive activation of Akt in all gastric cancer cell lines tested. These results suggest that the overexpression of $FLIP_S$ in gastric cancer cells may be correlated with the constitutive activation of Akt and consequently with the TRAIL-resistance.

PI3K/Akt signaling regulates $FLIP_S$ expression and sensitivity to TRAIL-induced cell death. In order to confirm the relationship between PI3K/Akt activity and FLIP expression in gastric cancer cells, we investigated whether the blockage of constitutive Akt activity by PI3K inhibitor altered *FLIP* gene expression (Fig. 4a). Akt activity was blocked by cycloheximide (1 μg/ml) or LY294002 (50 μ*M*) treatment in SNU-216 cells. However, changes in Akt activity were not detected in SNU-668 cells, probably because of the low basal Akt activity. $FLIP_S$ expression in SNU-216 and SNU-668 was reduced by cycloheximide or LY294002 treatment. In contrast, the expression of $FLIP_L$ was unaffected. To determine whether these decreases in $FLIP_S$ protein levels were associated with the suppression of its tran-

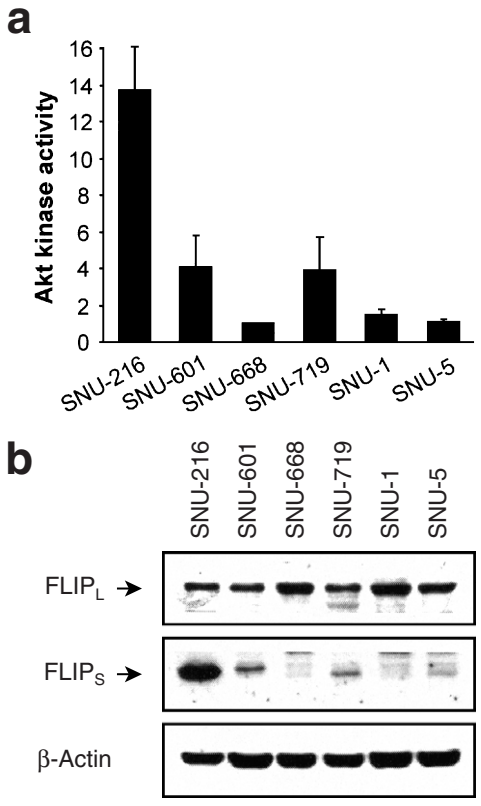

Fig. 3. Constitutive Akt activity and the expression levels of FLIP in human gastric cancer cell lines. (a) Constitutive Akt activity was measured by kinase assay. The relative activities compared with that in SNU-668, which showed the lowest activity of Akt. Values represent the means±SD of three experiments. (b) The expression levels of FLIP were detected by immunoblotting with anti-$FLIP_L$ and anti-$FLIP_S$ antibodies. β-Actin was used as the internal standard.

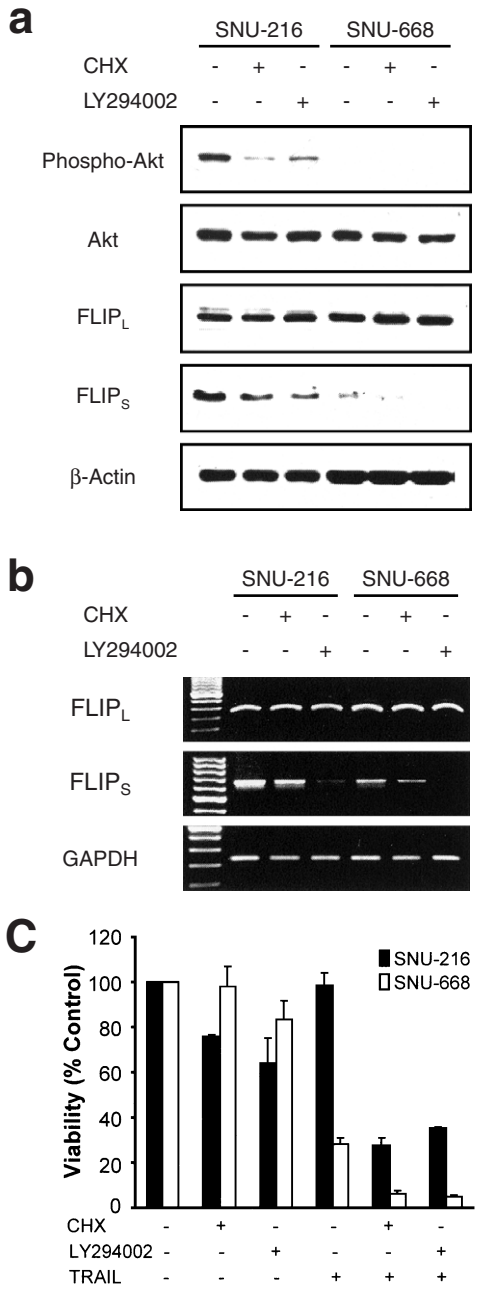

Fig. 4. Effects of PI3K/Akt inhibition on $FLIP_S$ expression and the cytotoxicity of TRAIL. SNU-216 and SNU-668 cells were treated with LY294002 (50 μ*M*) or CHX (1 μg/ml) for 24 h. (a) Western blotting was performed with anti-phospho-Akt, anti-Akt, anti-$FLIP_L$, or anti-$FLIP_S$ antibodies. (b) $FLIP_L$, $FLIP_S$, and GAPDH mRNA levels were analyzed by RT-PCR. Product sizes were 227 bp for $FLIP_L$, 666 bp for $FLIP_S$, and 233 bp for GAPDH (internal standard). (c) SNU-216 and SNU-668 cells were pretreated with CHX (1 μg/ml) or LY294002 (50 μ*M*) for 1 h, and then treated with TRAIL (100 ng/ml) for 24 h. Data represent means±SD (n=4).

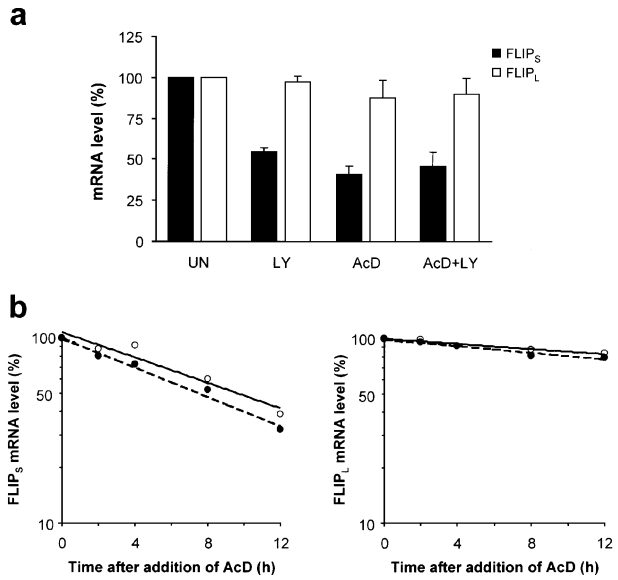

Fig. 5. Effects of actinomycin D (AcD) on LY294002-mediated FLIP$_S$ downregulation. (a) SNU-216 cells were treated with AcD (2.5 μg/ml) for 24 h in the presence or absence of LY294002 (50 μ*M*). Quantitative RT-PCR was performed with specific primers. (b) Effect of LY294002 on the half-lives of FLIP$_S$ mRNA and FLIP$_L$ mRNA. SNU-216 was incubated in the presence (filled circles and broken line) or absence (open circles and solid line) of LY294002 (50 μ*M*) for 24 h before the addition of AcD (2.5 μg/ml) for the indicated times to block transcription. Total RNA was extracted from the cells at the indicated times after administration of AcD. The decay of FLIP$_S$ mRNA and FLIP$_L$ mRNA was monitored by quantitative RT-PCR. GAPDH mRNA level was used as an internal control. The relative amount was expressed as a percentage of the value at 0 h and plotted on a logarithmic scale. Data represent means±SD (*n*=3).

scriptional level, FLIP$_S$ mRNA levels were examined by RT-PCR (Fig. 4b). Treatment with either cycloheximide or LY294002 resulted in reduced FLIP$_S$ mRNA levels, but not reduced levels of FLIP$_L$ mRNA. Based on these results, we speculated that the suppression of Akt activity by cycloheximide or LY294002 downregulated FLIP$_S$ expression in SNU-216 cells.

In order to examine the role of PI3K/Akt in the control of TRAIL-sensitivity in gastric cancer cell lines, SNU-216 and SNU-668 cells were pretreated with cycloheximide (1 μg/ml) or LY294002 (50 μ*M*) for 1 h, and then treated with TRAIL (100 ng/ml) for 24 h (Fig. 4c). In both cell lines, cell death was enhanced by combined treatment with TRAIL and cycloheximide or LY294002. Our results showed that the inhibition of Akt activity with cycloheximide or LY294002 potentiated the cell death caused by TRAIL, regardless of the original sensitivity of the cell lines to TRAIL. These results suggest that the downregulation of FLIP$_S$ expression by the inhibition of Akt activity might be responsible for the TRAIL-sensitivity induced by the combined treatment with TRAIL and LY294002.

Mechanism of FLIP$_S$ regulation by Akt activity. To determine whether the downregulation of FLIP$_S$ mRNA by LY294002 was attributable to reduced RNA synthesis, we examined by effect of actinomycin D treatment, using the real-time PCR method. Treatment with actinomycin D (2.5 μg/ml) alone for 24 h did not cause cell death and decreased the basal mRNA level of FLIP$_S$. Actinomycin D plus LY294002 did not cause enhanced reduction of FLIP$_S$ mRNA expression compared to the reduction induced by LY294002 alone. Actinomycin D did not affect the transcription of FLIP$_L$ mRNA in the presence or absence of LY294002 (Fig. 5a). These findings indicate that LY294002 may act to decrease the transcription of FLIP$_S$ mRNA.

In addition, to determine whether LY294002 decrease the stability of FLIP$_S$ mRNA or FLIP$_L$ mRNA, the half-lives of

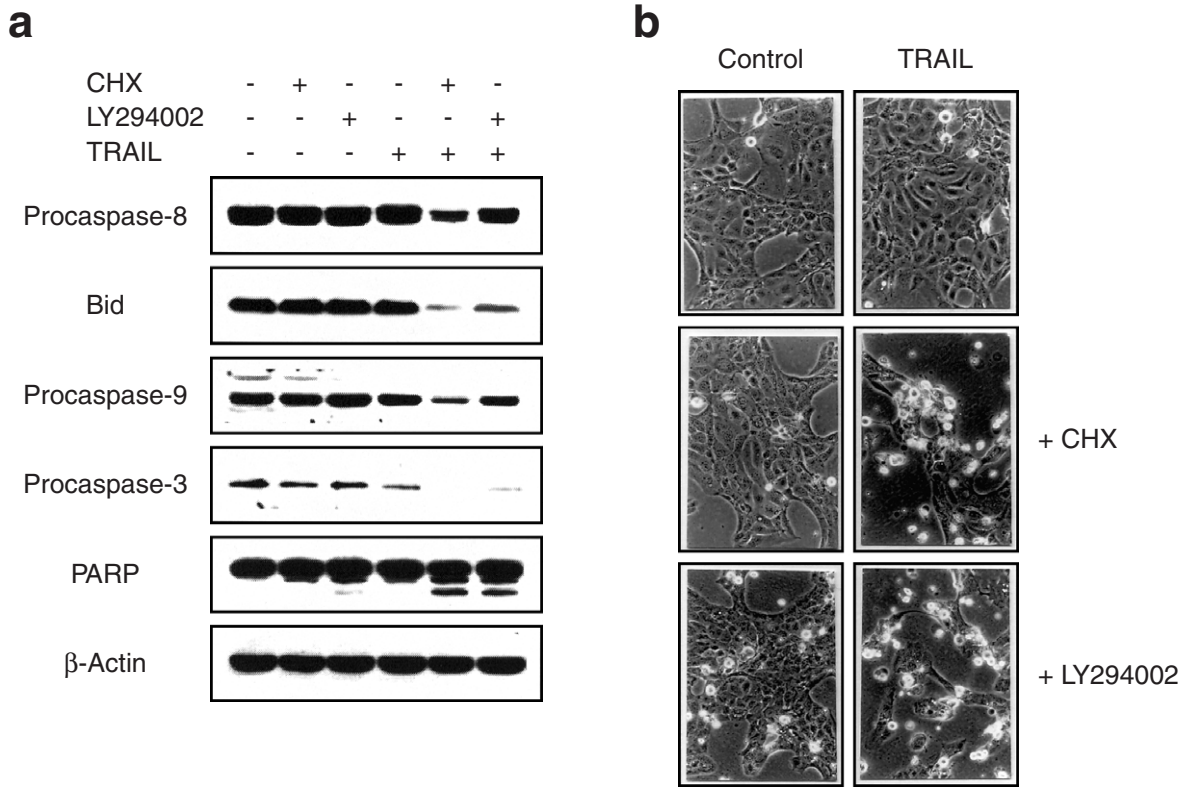

Fig. 6. Activation of caspases, PARP cleavage and morphological changes by CHX plus TRAIL or by LY294002 plus TRAIL in SNU-216 cells. SNU-216 cells were pretreated with CHX (1 μg/ml) or LY294002 (50 μ*M*) for 1 h, and then TRAIL (100 ng/ml) was applied to both for 24 h. (a) The activations of caspases, Bid and PARP cleavage were determined by western blot analysis. (b) Morphological alterations were recorded from phase-contrast microscopic observations.

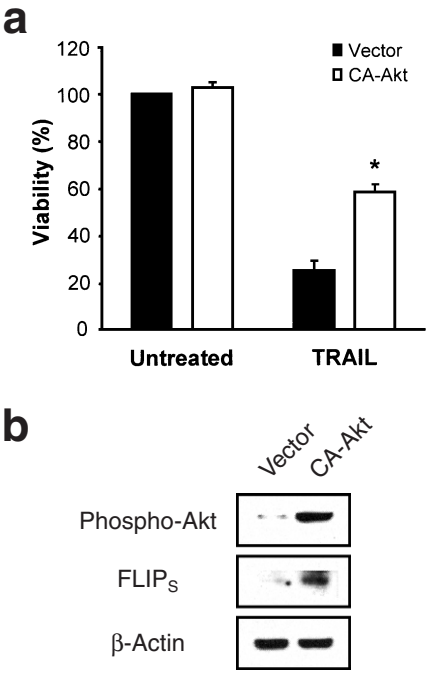

Fig. 7. The overexpression of constitutively active Akt inhibited the sensitivity of the SNU-668 cell line to TRAIL. SNU-668 cells were transiently transfected with constitutively active Akt (CA-Akt) or an empty vector. (a) Cells were treated with or without TRAIL (100 ng/ml) for 24 h. Cell survival was determined by crystal violet assay. Data represent means±SD (n=4). * P<0.001 compared to the empty vector. Statistical significance of differences was determined using the Newman-Keuls multiple comparison test. (b) Immunoblot analysis was performed for phospho-Akt and $FLIP_S$.

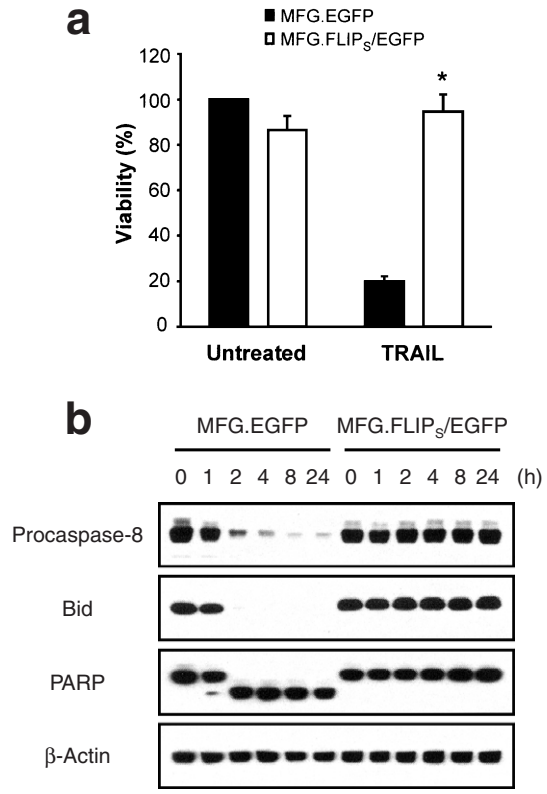

Fig. 8. $FLIP_S$ expression confers resistance to TRAIL-mediated apoptosis. SNU-668 cells were infected with either MFG.EGFP or MFG.$FLIP_S$/EGFP. (a) Cells were cultured with or without TRAIL (100 ng/ml) in fresh medium for 24 h and then cell survival was determined by crystal violet assay. Data represent means±SD (n=4). * P<0.001 relative to MFG.EGFP. Statistical significance was determined using the Newman-Keuls multiple comparison test. (b) After TRAIL treatment in a time-dependent manner, caspase-8 activation, Bid and PARP cleavage were determined by western blot analysis.

$FLIP_S$ and $FLIP_L$ mRNA were examined by monitoring mRNA decay after actinomycin D treatment. SNU-216 was pretreated with LY294002 (50 μ*M*) for 24 h, and then treated with actinomycin D (2.5 μg/ml) for the various times to block transcription. The decay of the $FLIP_S$ mRNA or $FLIP_L$ mRNA was monitored by real-time PCR (Fig. 5b). Following the inhibition of transcription by actinomycin D, the loss of LY294002-induced $FLIP_S$ mRNA was rapid and was not significantly different from that observed in cells treated with actinomycin D alone. However, actinomycin D slightly decreased $FLIP_L$ mRNA level in the presence or absence of LY294002 pretreatment. Thus, we concluded that LY294002-mediated reduction of $FLIP_S$ mRNA was regulated at the transcriptaional level independently of mRNA stability.

The inhibition of PI3K/Akt activity enhances TRAIL-induced apoptosis by caspases activation. We further examined whether treatment with PI3K inhibitor along with TRAIL affects the activation of caspases in SNU-216. In particular, we assessed the effects of treatment with LY294002 (50 μ*M*) or cycloheximide (1 μg/ml) for 24 h on caspase-8, Bid cleavage, caspase-9, and caspase-3 activation in the presence or absence of TRAIL (100 ng/ml) (Fig. 6a). In SNU-216 cells, TRAIL alone did not induce a significant level of proteolytic processing of procaspase-8, whereas the combination of TRAIL and LY294002 or cycloheximide induced the proteolytic processing of procaspase-8. Similar results were observed for the Bid cleavage and activation of caspase-9 and caspase-3. Additional studies demonstrated that TRAIL alone failed to induce PARP cleavage, whereas the combination of TRAIL and LY294002 or cycloheximide caused PARP cleavage, yielding a characteristic 85 kDa fragment. These observations are consistent with the morphological features (Fig. 6b). Cells undergoing apoptosis during treatment with combined TRAIL and LY294002 or cycloheximide showed cell surface blebbing and the formation of apoptotic bodies. The results consistently showed that the inhibition of Akt activity significantly enhanced TRAIL-induced apoptosis through the activation of caspases.

Overexpression of constitutively active Akt and $FLIP_S$ blocks TRAIL-mediated apoptosis in SNU-668 cells. The potentiating effect of LY294002 on TRAIL-induced apoptosis suggested that Akt signaling might be important for TRAIL-resistance in gastric cancer cells. In order to confirm our observations on the role of Akt in TRAIL-induced apoptosis, we transfected a vector containing constitutively active Akt or an empty vector into the TRAIL-sensitive cell line SNU-668, which was then subjected to TRAIL treatment (100 ng/ml) for 24 h. The overexpression of constitutively active Akt blocked apoptosis mediated by TRAIL in SNU-668 cells (Fig. 7a). To confirm that Akt activity inhibits TRAIL-induced cell death through regulation of $FLIP_S$, $FLIP_S$ protein level was analyzed using western blotting (Fig. 7b).

Furthermore, to examine the role of $FLIP_S$ expression in the control of TRAIL-mediated apoptosis in gastric cancers, SNU-668 cells were infected with either retroviral vector MFG.EGFP or MFG.$FLIP_S$/EGFP. Overexpression of $FLIP_S$ completely protected cells against TRAIL-mediated cell death (Fig. 8a). In addition, we assessed whether $FLIP_S$ inhibits TRAIL-induced apoptosis by blocking cleavage of procaspase-8. As shown in Fig. 8b, caspase-8 activation, Bid and PARP cleavage mediated by TRAIL were completely blocked by exogenous $FLIP_S$. These results corroborated the conclusion that upregulation of

$FLIP_S$ by Akt is involved in the resistance of human gastric cancer cells to TRAIL-induced apoptosis.

Discussion

In the present study, we found that human gastric carcinoma cell lines respond differently to TRAIL. Although most of the cell lines were TRAIL-sensitive to various degrees, SNU-216 was highly resistant. Thus, we examined the intracellular mechanism responsible for the different TRAIL-sensitivities in human gastric cancer cell lines. We found that, in human gastric cancer cells, the level of active Akt is closely associated with the inhibition of TRAIL-induced cell death, and that Akt activation regulates the expression of $FLIP_S$, but not that of $FLIP_L$. Therefore, this study demonstrates that Akt may control the cytotoxic effect of TRAIL by modulating $FLIP_S$ expression in human gastric cancers. To our knowledge, this is the first report on the role of the Akt/$FLIP_S$ pathway in the control of TRAIL-induced apoptosis in human gastric cancer cells.

It has been reported that the inhibition of protein synthesis enhances cell death induced by death-inducing ligands by down-regulating FLIP expression.[18, 32] In the present study, TRAIL-sensitivity of the SNU-216 cell line, which is highly resistant to TRAIL, was increased by cycloheximide treatment. In the other cell lines tested in this study, TRAIL-sensitivity was also enhanced by cycloheximide. These results suggest that TRAIL-resistance in human gastric cancer cell lines is, at least in part, attributable to cycloheximide-sensitive antiapoptotic molecules.

Previously, it has been shown that chemotherapeutic agents and ionizing radiation can enhance TRAIL-induced cytotoxicity.[33–36] Such synergistic effects of combined treatment with TRAIL and chemotherapeutic agents or ionizing radiation may result from a decrease in the intracellular levels of antiapoptotic molecules or from an increase in the expression of TRAIL receptors.[33, 35–37] To identify the factors responsible for TRAIL-resistance in the SNU-216 cell line, we first determined the expression levels of the TRAIL receptors and of the death-inducing complex molecule, FADD. Our results, as shown in Fig. 2, demonstrated that the expression levels of TRAIL receptors and FADD were variable in individual gastric cancer cell lines tested and did not correlate with TRAIL-resistance.

Recently, it was reported that elevated Akt activity was related to resistance to TRAIL-induced apoptosis in some cancers including prostate cancer and renal cancer.[14–17] Previous reports suggested that TRAIL-resistance involving Akt activity appears to be mediated through the inhibition of Bid cleavage.[14, 15] To confirm this hypothesis in gastric cancer cells, we measured the levels of the activated form of Akt and Bid cleavage, and found that the TRAIL-resistant cell line SNU-216 had the highest level of the activated form of Akt. Since the inhibition of constitutive Akt activity by treatment with the PI3K inhibitor LY294002 sensitized SNU-216 cells to TRAIL, we suggest that TRAIL-resistance via the activation of Akt is dependent upon the PI3K/Akt pathway. In addition, we were able to detect Bid cleavage after treating SNU-216 cells with TRAIL plus LY294002. Previous reports showed that caspase-8 activation was induced by TRAIL alone in both TRAIL-resistant and TRAIL-sensitive prostate cancer cell lines, but Bid cleavage was induced only by combined treatment with TRAIL and PI3K inhibitor in a TRAIL-resistant cell line. It was suggested that active Akt regulated the level of Bid cleavage downstream of caspase-8.[15] In comparison, in the present study, caspase-8 activation in SNU-216 was detected after treatment with TRAIL plus LY294002, but not after treatment with TRAIL alone. Our data suggest that Akt signaling protects gastric cancer cells against TRAIL-induced apoptosis by regulation upstream of caspase-8.

To investigate the relationship between FLIP expression and TRAIL-resistance in gastric cancer cell lines, we tested the expression levels of splice variants of FLIP ($FLIP_L$ and $FLIP_S$). In the present study, we found that $FLIP_L$ was expressed in all cell lines tested at similar levels. In comparison, $FLIP_S$ was overexpressed in SNU-216, which suggested that this high expression level of $FLIP_S$ might be related with TRAIL-resistance in this cell line. It has been reported that $FLIP_L$ and $FLIP_S$ inhibit different steps of caspase-8 activation.[19] $FLIP_L$ allows partial cleavage of procaspase-8, generating a p10 subunit, which is an active fragment of caspase-8. In comparison, $FLIP_S$ completely inhibits the cleavage of caspase-8. We speculate that the anti-apoptotic function of $FLIP_S$ may be more potent than that of $FLIP_L$ in TRAIL-induced apoptosis in the case of human gastric cancer cells.

It has been reported that $FLIP_S$ and $FLIP_L$ are differently regulated,[38, 39] although the difference in the regulatory mechanisms remains unclear. Our data demonstrated that Akt activity regulated only $FLIP_S$, but not $FLIP_L$ expression, and this may contribute to the TRAIL-resistance in human gastric cancer cells. In particular, the Akt activity affected the transcriptional level of $FLIP_S$, but not the mRNA stability. Our finding was confirmed by the overexpression of constitutively active Akt in the TRAIL-sensitive SNU-668 cells, which showed upregulation of $FLIP_S$ expression and suppression of apoptosis induced by TRAIL. Moreover, overexpression of $FLIP_S$ rendered SNU-668 cells resistant to TRAIL by blocking the activation of caspase-8. These observations show that resistance to TRAIL is, at least in part, regulated by Akt/$FLIP_S$ signaling in human gastric cancer, although the mechanism through which the PI3K/Akt signaling pathway differentially regulates $FLIP_L$ and $FLIP_S$ expression in human gastric cancer needs to be elucidated. We believe that these results will help to provide an understanding of the mechanism of TRAIL-resistance in gastric cancer cells, and provide important clues for the design of future studies on TRAIL-based gastric cancer therapies.

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