Phosphoinositide 3-kinase/Akt and nuclear factor κB pathways are involved in tumor necrosis factor-related apoptosis-inducing ligand resistance in human gastric cancer cells

JING LIU, XIUJUAN QU, LING XU, YE ZHANG, JINGLEI QU, KEZUO HOU and YUNPENG LIU

Department of Medical Oncology, The First Hospital of China Medical University, Shenyang 110001, P.R. China

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Abstract. Human gastric cancer cells are generally believed to be less sensitive to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, but the events responsible for this resistance are as yet unclear. In this study, we investigated the role of the phosphoinositide 3-kinase (PI3K)/Akt, nuclear factor κB (NF-κB) and extracellular signal-regulated kinase (ERK) signaling pathways in the TRAIL resistance of gastric cancer cells. TRAIL failed to induce observable apoptosis in the three cell lines. Further investigation revealed that TRAIL engagement led to the activation of PI3K/Akt as well as of NF-κB. The inhibition of PI3K/Akt by a specific inhibitor facilitated TRAIL-induced apoptosis. Blockage of TRAIL-induced NF-κB activation by transient transfection with a phosphorylation-defective mutant IκB also enhanced the sensitivity of cells towards TRAIL. Meanwhile, ERKs were highly activated in the resting cells and were not further activated by TRAIL treatment. However, the inhibition of ERK activity by PD98059 also enhanced the apoptosis-inducing ability of TRAIL. Our data demonstrated that the activation of PI3K/Akt and NF-κB by TRAIL is responsible for resistance to TRAIL in human gastric cancer cells. Blockage of survival signals significantly enhances the apoptosis induced by TRAIL.

Introduction

Gastric cancer is one of the most common malignancies worldwide, particularly in eastern Asian countries such as China, Japan and Korea (1). Although combinational chemotherapy has brought about some improvements, the prognosis in cases of advanced disease remains very poor (2). A truly effective regimen for the treatment of advanced gastric cancer has not yet been established.

Recently, a biological agent in the form of a monoclonal antibody exhibited significant therapeutic benefits in the treatment of breast, lung and colon cancer (3-5). These results confirm that biological therapy is a promising method for cancer treatment. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily, which induces apoptosis through the engagement of death receptors. Unlike other members of the TNF family, TRAIL preferentially induces apoptosis in a variety of tumor cells, whereas it has only slight toxicity towards most normal cells and induces a mild inflammatory response (6,7). This selective toxicity for cancer cells and induction of low-level inflammation make TRAIL a potential tumor-specific cancer therapy. Clinical trials using agonistic monoclonal antibodies specific for TRAIL receptors (DR4 or DR5) have achieved promising results in patients with advanced solid malignancies (8,9).

Despite the powerful activity of TRAIL towards most types of cancer cells, some tumors display intrinsic or acquired resistance to TRAIL-induced apoptosis (10,11). Previous studies by our group and others have shown that gastric cancer cells are less sensitive towards TRAIL-induced apoptosis (12-14). A thorough understanding of the mechanisms involved in TRAIL resistance is very important to overcome the resistance and facilitate the clinical use of TRAIL. TRAIL resistance can occur at different steps in the signaling cascade, and may involve several mechanisms. Some signaling pathways that are critical for cell proliferation and survival, such as the phosphoinositide 3-kinase (PI3K)/Akt, nuclear factor κB (NF-κB) and extracellular signal-regulated kinase (ERK) pathways, are also involved in TRAIL-mediated apoptosis. It has been reported that highly active PI3K/Akt signaling is associated with TRAIL resistance, and blockage of the PI3K/Akt pathway reverses the resistance to TRAIL in certain primary resistant tumor cells (15,16). However, the NF-κB and ERK pathways play diverse roles in TRAIL-induced apoptosis. NF-κB activation is usually thought to have an anti-apoptotic effect. It maintains TRAIL resistance in human pancreatic cancer cells, but does not modulate the sensitivity of renal carcinoma cells to TRAIL (17,18). In melanoma cells, the activation of ERK has been reported to protect against TRAIL-induced apoptosis;

Correspondence to: Dr Yunpeng Liu, Department of Medical Oncology, The First Hospital of China Medical University, Shenyang 110001, P.R. China
E-mail: cmuliuyunpeng@yahoo.cn

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however, in lung cancer cells the activation of ERK is required for PG490-mediated sensitization to TRAIL (19,20). These controversial results suggest that the role of each signaling pathway in TRAIL-induced apoptosis in different tumors is in need of further individual study.

To further investigate the associated molecules involved in TRAIL resistance, in the present study we investigated the effects of TRAIL on the activities of the PI3K/Akt, NF-κB and ERK pathways, as well as the role they play in TRAIL-mediated apoptosis in three human gastric cancer cell lines. Our data demonstrated that these survival signals interrupt TRAIL-induced apoptosis. Individual inhibition of any of these signals may partially reverse resistance to TRAIL-induced apoptosis.

Materials and methods

Cell cultures. The human gastric cancer cells MGC803, BGC823 and SGC7901 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37˚C under an atmosphere of 95% air and 5% CO_2. The cells were routinely subcultured every 2-3 days, and were all from the logarithmic phase of growth.

Reagents and antibodies. Recombinant human TRAIL/Apo2L was from Cytolab/Peprotech Asia (USA). LY294002, PD98059 and DiOC6 were purchased from Sigma-Aldrich (USA). Anti-phospho-Akt (Ser-473), anti-Akt, anti-phospho-ERK, anti-ERK, anti-IκBα and anti-tubulin antibodies were purchased from Santa Cruz Biotechnology (USA).

Cell viability assay. Cell viability was measured using a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded at $5x10^4$ cells/well in 96-well plates, incubated overnight and then exposed to the indicated concentrations of TRAIL for the indicated times. Thereafter, 20 µl of MTT solution (5 mg/ml) was added to each well, and the cells were incubated for another 4 h at 37˚C. After removal of the culture medium, the cells were lysed in 200 µl of dimethylsulfoxide (DMSO), and the optical density (OD) was measured at 570 nm with a microplate reader (Model 550; Bio-Rad Laboratories, USA). The following formula was used: cell viability = (OD of the experimental sample/OD of the control group) x 100%.

Analysis of apoptosis. Cells were seeded at $3x10^5$ cells/well in 6-well plates, incubated overnight and then exposed to the indicated concentrations of TRAIL for the indicated times. The cells were then collected and washed twice with phosphate-buffered saline (PBS). After being fixed with ice-cold 70% ethanol for 12 h, the samples were washed twice with PBS and then incubated with 20 µg/ml RNase A at 37˚C and 10 µg/ml propidium iodide for 30 min in the dark. Finally, the samples were evaluated by flow cytometry, and the data were analyzed by WinMDI software.

Western blotting. Cells were washed twice with ice-cold PBS and solubilized in 1% Triton lysis buffer [1% Triton X-100, 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and 2 µg/ml aprotinin] on ice, then quantified using the Lowry method. Cell lysate proteins (50 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (Immoblin-P; Millipore, USA). The membranes were blocked with 5% skim milk in TBST buffer [10 mM Tris (pH 7.4), 150 mM NaCl and 0.1% Tween-20] at room temperature for 1 h and incubated overnight at 4˚C with the indicated primary antibodies. After the membranes were washed with TBST buffer, they were reacted with the appropriate horseradish peroxidase-conjugated secondary antibodies for 30 min at room temperature. After extensive washing with TBST buffer, the proteins were visualized with enhanced chemiluminescence reagent (SuperSignal Western Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA).

Transient transfection. Transient transfection was performed using Lipofectamine 2000 reagent (Invitrogen, USA), according to the manufacturer's instructions. Briefly, the cells were seeded in 6-well plates at a density of $5x10^5$ cells/well and incubated for 24 h. Before transfection, Lipofectamine (6 µl) was diluted dropwise into OPTI-MEM and incubated at room temperature for 5 min. Then, 2 µg of empty vector or hemagglutinin-tagged phosphorylation-defective mutant IκB expression constructs were added to the diluted Lipofectamine and incubated for another 20 min before being dropped into the medium. After 48 h of transfection, the cells were subcultured for further use.

Statistical analysis. The experiments were repeated at least three times. Data are expressed as the means ± SD. Differences in the results for two groups were evaluated by the Student's t-test. $P<0.05$ was considered to be statistically significant.

Results

Human gastric cancer cells are resistant to TRAIL-induced apoptosis. Three human gastric cancer cell lines, MGC803, BGC823 and SGC7901, were exposed to 1-1,000 ng/ml TRAIL for 24 or 48 h. As shown in Fig. 1A, the inhibitory effects of TRAIL on the proliferation of the three cell lines reached the plate phase at 100 ng/ml. Compared with the untreated control, TRAIL at 100 ng/ml reduced cell viability to 70.67±5.13% in MGC803 cells, to 93.67±3.21% in BGC823 cells, and to 99.67±2.52% in SGC7901 cells, respectively. Even if the dose of TRAIL was increased to 1,000 ng/ml or the exposure prolonged to 48 h, there was no further increase in cytotoxicity (Fig. 1B). Flow cytometric analysis showed that 100 ng/ml TRAIL induced <8% apoptosis in the three cell lines (Fig. 1C), whereas the same concentration of TRAIL induced >90% apoptosis in TRAIL-sensitive Jurkat T cells (data not shown). These data indicate that gastric cancer cells are resistant to TRAIL-triggered apoptosis.

The PI3K/Akt signaling pathway is activated by TRAIL and prevents cells from undergoing apoptosis. To determine the

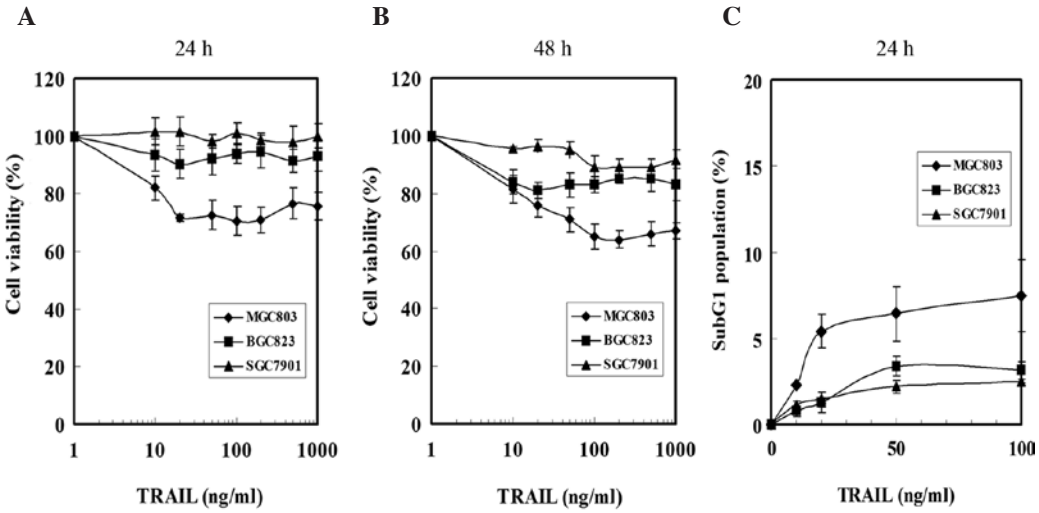

Figure 1. Effect of TRAIL on the cell viability of human gastric cancer cells. Human gastric cancer cells MGC803, BGC823 and SGC7901 were exposed to 1-1,000 ng/ml TRAIL for 24 (A) or 48 h (B), and cell viability was measured by the MTT assay. Data are the means ± SD of three independent experiments performed in triplicate. (C) The cells were exposed to 0-100 ng/ml TRAIL for 24 h, and the apoptosis was analyzed by flow cytometry following staining with propidium iodide. Data are the means ± SD of three independent experiments.

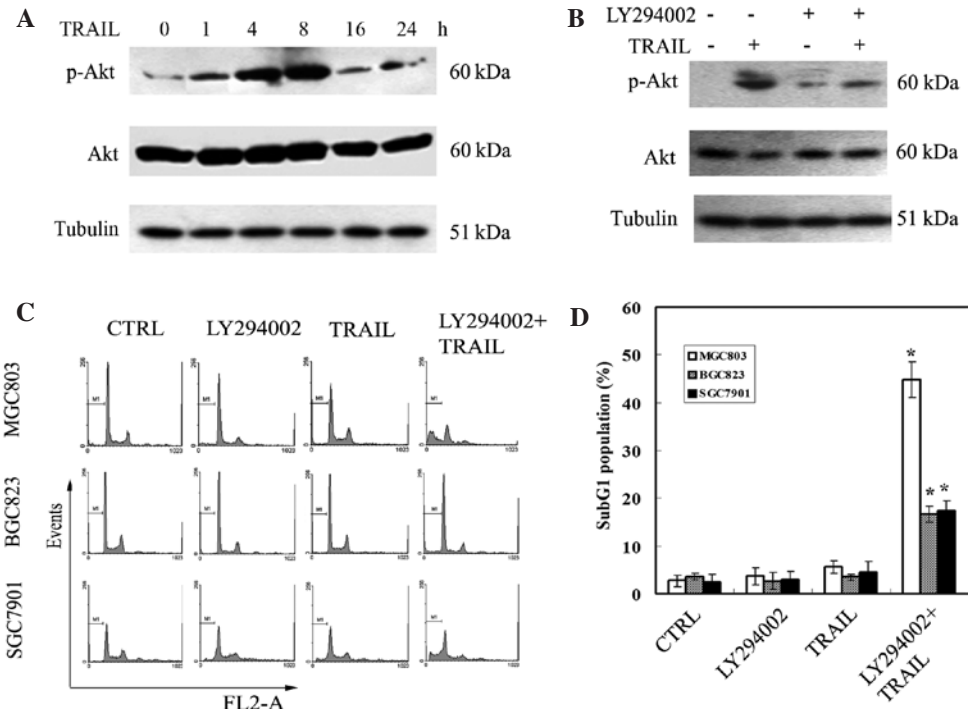

Figure 2. The PI3K/Akt signaling pathway is activated by TRAIL and prevents cells from undergoing apoptosis. (A) SGC7901 cells were exposed to 100 ng/ml TRAIL for the indicated time, and the level of phospho(p)-Akt was detected by Western blotting. (B) SGC7901 cells were pre-treated with 50 μM LY294002 for 1 h, then exposed to 100 ng/ml TRAIL for an additional 8 h, and the level of phospho-Akt was determined by Western blotting. The total Akt and tubulin were used as the internal loading control. Blots are representative of three independent experiments. (C and D) MGC803, BGC823 and SGC7901 cells were pre-treated with 50 μM LY294002 for 1 h, then exposed to 100 ng/ml TRAIL for an additional 24 h, and the apoptosis was detected by flow cytometry following propidium iodide staining. Histograms are representative of three independent experiments, and the columns are the means ± SD of three independent experiments. *P<0.05 vs. cells treated with TRAIL alone.

role of the PI3K/Akt signaling pathway in TRAIL-induced apoptosis, we investigated Akt activation in the most resistant SGC7901 cells after treatment with 100 ng/ml TRAIL for 1-24 h. Upon TRAIL treatment, the phosphorylation of Akt increased at 1 h, reached a peak at 8 h, declined, and thereafter remained at the basal level (Fig. 2A). Pre-treatment with LY294002, the specific inhibitor of PI3K, followed by exposure to TRAIL reduced the phosphorylation of Akt at 8 h (Fig. 2B). We next investigated whether the inhibition of Akt activity affects TRAIL-induced apoptosis. As shown in Fig. 2C and D, TRAIL or LY294002 alone induced <5% apoptosis in the three cell lines, whereas pre-treatment

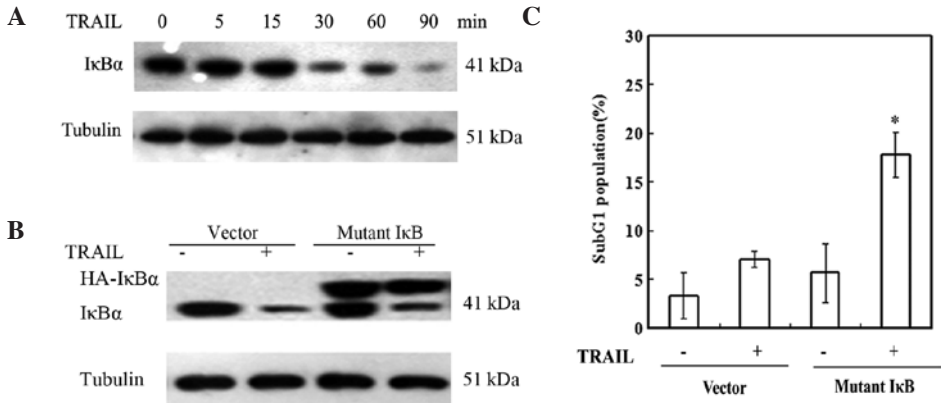

Figure 3. TRAIL-triggered rapid activation of NF-κB contributes to the resistance towards TRAIL. (A) SGC7901 cells were exposed to 100 ng/ml TRAIL for the indicated time, and the level of IκB was detected by Western blotting. (B) SGC7901 cells were transiently transfected with an empty vector or a cDNA encoding phosphorylation-defective mutant IκB for 48 h, and then exposed to 100 ng/ml TRAIL for 30 min. The degradation of IκB was analyzed by Western blotting. Tubulin was used as the internal loading control. Blots are representative of three independent experiments. (C) After transient transfection for 48 h, the SGC7901 cells were treated with 100 ng/ml TRAIL for 24 h, and the apoptosis was analyzed by flow cytometry following propidium iodide staining. Columns are the means ± SD of three independent experiments. *P<0.05 vs. cells transfected with the empty vector and treated with TRAIL alone.

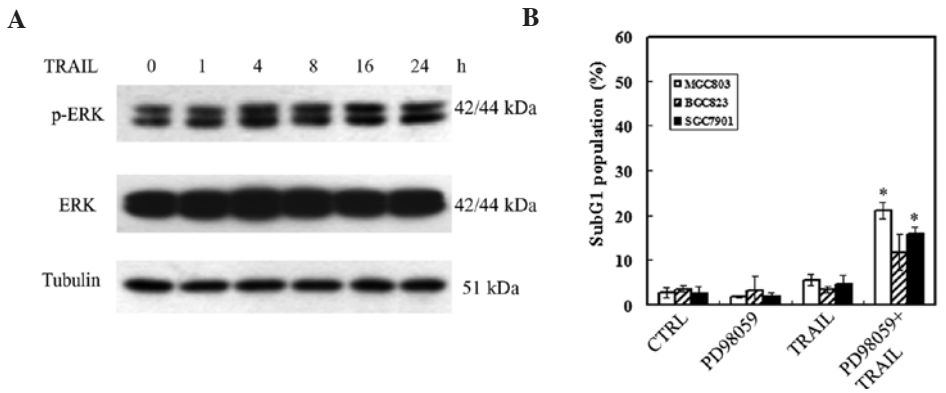

Figure 4. Inhibition of the ERK pathway also increases the sensitivity of cells to TRAIL-induced apoptosis. (A) SGC7901 cells were exposed to 100 ng/ml TRAIL for the indicated time, and the level of phospho(p)-ERK was detected by Western blotting using specific antibodies. (B) MGC803, BGC823 and SGC7901 cells were pre-treated with 20 μM PD98059 for 1 h, then exposed to 100 ng/ml TRAIL for an additional 24 h. The apoptosis was analyzed by flow cytometry following propidium iodide staining. Columns are the means ± SD of three independent experiments. *P<0.05 vs. cells treated with TRAIL alone.

with LY294002 followed by TRAIL exposure significantly increased the apoptotic population to 44.73±3.75% in MGC803 cells, to 17.38±2.14% in SGC7901 cells, and to 16.62±1.68% in BGC823 cells, respectively (P<0.05). These data indicate that the TRAIL-induced activation of the PI3K/Akt pathway is responsible for the resistance of cells towards TRAIL-induced apoptosis.

TRAIL-triggered rapid activation of NF-κB contributes to resistance towards TRAIL. We next investigated the role of NF-κB signaling in TRAIL-induced apoptosis. In SGC7901 cells, TRAIL triggered rapid activation of NF-κB, as evidenced by the clear degradation of IκB at as early as 30 min, maintained up to 90 min after TRAIL treatment (Fig. 3A). SGC7901 cells were then transiently transfected with either an empty vector or a cDNA encoding a phosphorylation-defective mutant IκB. The mutant IκB protein did not undergo degradation at 30 min upon TRAIL treatment, which indicated that the NF-κB signaling pathway was inactivated in the cells overexpressing mutant IκB (Fig. 3B). Analysis of apoptosis indicated that, in the cells transfected with the empty vector, TRAIL induced only 7.09±0.84% apoptosis. By contrast, in the cells overexpressing mutant IκB, TRAIL-triggered apoptosis was increased to 17.80±2.34% (P<0.05) (Fig. 3C). These data indicate that the rapid activation of NF-κB by TRAIL protects gastric cancer cells from TRAIL-induced apoptosis, and the inactivation of the NF-κB pathway enhances the apoptosis-inducing ability of TRAIL.

Inhibition of the ERK pathway increases the sensitivity of cells to TRAIL. Unlike the PI3K/Akt and NF-κB signaling pathways, ERK was already highly activated in the resting SGC7901 cells, and the incubation of the cells with TRAIL for 1-24 h did not further enhance the activity of ERK (Fig. 4A). However, pre-treatment with PD98059, a specific inhibitor of ERK signaling, also enhanced the TRAIL-induced apoptosis. As shown in Fig. 4B, TRAIL or PD98059 alone induced <5% apoptosis in the three cell lines, whereas in the presence of PD98059, the apoptosis induced by TRAIL was increased to 21.18±1.75% in MGC803 cells, to 15.7±1.9% in SGC7901

cells, and to 11.86±4.00% in BGC823 cells, respectively ($P<0.05$). These data indicate that although TRAIL has little effect on ERK activity, the high activity of ERKs at a basal level also interrupts TRAIL-triggered apoptosis.

Discussion

The resistance of gastric cancer cells towards TRAIL-induced apoptosis limits the use of TRAIL as a potent antitumor agent. Several attempts to conquer TRAIL resistance in gastric cancer cells have been made in the last couple of years. Usually, TRAIL induces obvious apoptosis in the most sensitive cells at a concentration under 100 ng/ml. In the present study, the inhibitory effect of TRAIL reached the plate phase at 100 ng/ml. Even when the dose of TRAIL was increased to 1,000 ng/ml or the exposure time prolonged to 48 h, cytotoxity was not further enhanced. This is consistent with results from other trials involving TRAIL-resistant tumor cells (21), and indicates that the cytotoxity of TRAIL on resistant cells cannot be enhanced simply by increasing the dose of TRAIL or by prolonging the exposure time. Other avenues must therefore be explored to overcome the resistance.

Recent studies have revealed that the down-regulation of anti-apoptotic molecules by other reagents enhances the apoptosis-inducing ability of TRAIL in gastric cancer cells (22-24). However, these studies overlooked a possibility which has been found in other tumor cells: that TRAIL activates certain survival signals so as to interrupt the apoptosis cascade (10). In the present study, we focused on the effect of TRAIL on PI3K/Akt, NF-κB and ERK, the three major signaling pathways involved in cell proliferation and survival. PI3K/Akt is a major signaling pathway that regulates cell survival, growth and apoptosis. It can be induced by a wide range of stimuli and often acts as an anti-apoptotic signal (25). Previous studies have shown that a high level of PI3K/Akt activity in cancer cells is responsible for TRAIL resistance (26-28). Nevertheless, little is known regarding whether TRAIL is capable of triggering further Akt activation in TRAIL-resistant tumor cells. Recently, Chen *et al* reported that TRAIL activated Akt in hepatocellular carcinoma cells and prevented cell death (29). Here, we provide evidence that TRAIL also activates PI3K/Akt in gastric cancer cells, and the inhibition of Akt activity significantly enhances the sensitivity of cells to TRAIL-induced apoptosis. These results indicate that TRAIL activates the PI3K/Akt signaling pathway, depressesing the apoptosis-inducing effect and leading to TRAIL resistance.

It has been reported that the engagement of TRAIL with its receptors activates NF-κB, which in turn initiates the transcription of several anti-apoptotic genes and protects cells from TRAIL-induced apoptosis (30). The inhibition of NF-κB activity increases the sensitivity of a variety of tumor cells towards TRAIL (17,31,32). However, in some cases, NF-κB inactivation fails to enhance the apoptosis triggered by TRAIL (18,33,34). Ishiguro *et al* reported that an ingredient of ginger, 6-gingerol, reduced the viability of gastric cancer cells by inhibiting NF-κB activation (22). However, as a herbal component 6-gingerol also has biological effects other than the blocking NF-κB activation, and the inhibition of NF-κB activity may be indirect and secondary to the other events. In the present study, the NF-κB signal was inactivated by the overexpression of a mutant IκB cDNA in SGC7901 cells. The inactivation of NF-κB was found to significantly enhance apoptosis in response to TRAIL. This is the first evidence that the specific inhibition of NF-κB activity enhances TRAIL-mediated apoptosis in gastric cancer cells, which indicates that, like the PI3K/Akt signal, NF-κB acts as an apoptotic inhibitor in gastric cancer cells.

ERKs are members of the MAP kinases, a superfamily of proteins that transmits signaling cascades from extracellular stimuli into cells. Usually, the activation of ERKs in response to death stimuli is believed to have an anti-apoptotic effect, based on the evidence of TRAIL-induced rapid ERK activation in certain tumor cell lines, and the inhibition of this activation sensitizes TRAIL-resistant tumor cells to TRAIL (19,35). On the other hand, ERK activation has been reported to have pro-apoptotic effects in TRAIL-mediated apoptosis in lung and prostate cancer cells (20,36). However, the role of ERK on TRAIL-mediated apoptosis in gastric cancer cells has not as yet been studied. Data from our study demonstrated that ERK was over-activated in the resting gastric cancer cells. Although TRAIL did not further activate ERK, the inhibition of ERK activity with a specific inhibitor significantly enhanced the apoptosis-inducing effect of TRAIL. This indicates that ERK signaling also protects gastric cancer cells from TRAIL-induced apoptosis.

Taken together, our data demonstrate that, in human gastric cancer cells, PI3K/Akt and NF-κB are activated by TRAIL and serve as survival signals to protect cells from TRAIL-induced apoptosis. A highly activated ERK signaling pathway is also involved in TRAIL resistance. Combined treatment of TRAIL with several inhibitors or with an inhibitor which has an effect on multiple signaling pathways may be a promising tool for the treatment of gastric cancer.

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