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Triptolide sensitizes lung cancer cells to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by inhibition of NF-κB activation

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Abbreviations: Apo2L, Apo2 ligand; DcR, decoy receptor; DR, death receptor; EMSA, electrophoretic mobility shift assay; FasL, Fas lignad; IκB, inhibitor kappa B; NF-κB, nuclear factor kappa B; TNFR1, TNF, tumor necrosis factor; TNFR1, TNF receptor 1; TRAIL, TNF-related apoptosis-inducing ligand.

Abstract

TNF-related apoptosis-inducing ligand (TRAIL/Apo-2L), a newly identified member of the TNF family promotes apoptosis by binding to the transmembrane receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5). TRAIL known to activate NF-κB in number of tumor cells including A549 (wt p53) and NCI-H1299 (null p53) lung cancer cells exerts relatively selective cytotoxic affects to the human tumor cell lines without much effect on the normal cells. We set out to identify an agent that would sensitize lung cancer cells to TRAIL-induced apoptosis through inhibition of NF-κB activation. We found that triptolide, an oxygenated diterpene extracted and purified from the Chinese herb *Tripterygium wilfordii* sensitized A549 and NCI-H1299 cells to TRAIL-induced apoptosis through inhibition of NF-κB activation. Pretreatment with MG132 which is a well-known NF-κB inhibitor by blocking degradation of IκBα also greatly sensitized lung cancer cells to TRAIL-induced apoptosis. Triptolide did not block DNA binding of NF-κB activated by TRAIL as in the case of TNF-α. It has been already proven that triptolide blocks transactivation of p65 which plays a key role in NF-κB activation. These observations suggest that triptolide may be a potentially useful drug to enhance TRAIL-induced tumor killing in lung cancer.

Keywords: antineoplastic agents; apoptosis; drug, Chinese herbal; lung neoplasms; NF-κB; tumor necrosis factor

Introduction

It is now well documented that loss of p53 function in certain cell types is associated with decreased sensitivity to chemotherapy and radiotherapy (Lowe *et al.*, 1993; Aas, *et al.*, 1996). Lung cancer that most frequently harbor p53 mutations is typically less responsive to chemotherapy compared to tumors that retain wild-type p53 (Fujiwara *et al.*, 1994; Levin, 1997). Strategies to overcome resistance to apoptosis are relevant to the new therapeutic approaches for lung cancer. Death receptors (DR)-mediated apoptosis is arising as an attractive anti-cancer strategy because death receptors initiate apoptosis regardless of p53 phenotype and also have direct access to the caspase machinery (Ashikenazi and Dixit, 1998). Best characterized death receptors are Tumor necrosis factor (TNF) receptor 1 (TNFR1) and Fas/CD95 (Smith *et al.*, 1994; Nagata, 1997). Ligands that activate these receptors are structurally related molecules belong to the TNF gene superfamily. Fas ligand (FasL)/CD95L binds to Fas/CD95 and TNF binds to TNFR1. The clinical utility of both TNF and FasL (CD95L), however, has been hampered by toxic side effects including NF-κB activation leading to septic shock and lethal hepatocyte apoptosis respectively.

TNF-related apoptosis-inducing ligand (TRAIL), or Apo2 ligand (Apo-2L) is a newly identified member of the TNF gene superfamily (Wiley *et al.*, 1995; Pitti *et al.*, 1996). TRAIL induces apoptosis of many transformed cell lines but not of normal cells, even though its death domain-containing receptors, DR4 and DR5, are expressed on both cell types (Pan *et al.*, 1997; Sheridon *et al.*, 1997). The resistant phenotype of normal cells to TRAIL-induced apoptosis appears to be due to selective expression of antagonistic decoy receptor (DcR1 and DcR2) in normal cells but not in tumor cells. A recent *in vivo* study demonstrated these selective tumor cell killing and normal cell protection by TRAIL in SCID mice model (Walczak *et*

al., 1997). At least, the matter of toxicity seems not to be major obstacle to the clinical utility of TRAIL.

Although it has been suggested that the expression of decoy receptors may determine the sensitivity of cancer cells to TRAIL-induced apoptosis, recent papers indicated that there is no correlation between TRAIL resistance and decoy receptor expression (Griffith and Lynch, 1998; Griffith *et al.*, 1998). At present, about 50% of the tumor cell lines tested so far seem to be sensitive to TRAIL-induced apoptosis (Griffith and Lynch, 1998) indicating that another half of the tumor cell lines might adopt resistant mechanisms to TRAIL-induced apoptosis. NF-κB activation which is a well known anti-apoptotic transcriptional factors in TNF-induced apoptosis is the most probable resistant mechanism. Initial investigations about TRAIL already showed that TRAIL could activate NF-κB through TRAIL receptors, even truncated DcR2 (Chaudhary *et al.*, 1997; Pan *et al.*, 1997).

We set out to identify compounds which sensitize lung cancer cells to TRAIL through inhibition of NF-κB. It has recently shown that PG490 (triptolide, molecular weight, 360), which is derived from Chinese herb *Tripterygium wilfordii*, cooperates with TNF to induce apoptosis in solid tumor cells through inhibition of NF-κB (Lee *et al.*, 1999). Here we demonstrate that TRAIL activates NF-κB in lung cancer cells and that triptolide sensitizes lung cancer cells to TRAIL-induced apoptosis through inhibiting NF-κB activation by TRAIL. Pretreatment with MG132 which is a well-known NF-κB inhibitor by blocking degradation of IκBα also greatly sensitizes lung cancer cells to TRAIL-induced apoptosis. Triptolide does not block DNA binding of NF-κB activated by TRAIL as in the case of TNF. It was already observed that triptolide blocks transactivation of p65 which plays a key role in NF-κB activation. These observations suggest that triptolide may be a potentially useful drug to enhance TRAIL-induced tumor killing in lung cancer.

Materials and Methods

Cells and reagents

A549 and NCI-H1299 cells were purchased from ATCC. Cells were cultured in appropriate media (F12-K in A549 and RPMI 1640 in NCI-H1299) with 10% fetal calf serum supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. Ttriptolide (PG490) was provided by Pharmagenesis (Palo Alto, CA). TRAIL was purchased from Alexis Biochemicals (San Diego, CA) and MG132 was obtained from Biomol Research Laboratories (Plymouth Meeting, PA).

Cell death assay

Cell viability was measured by an MTT assay. Briefly, untreated cells or cells treated with triptolide and/or TRAIL in a 96-well plate were harvested at the indicated times followed by the addition of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT) and then cells were solubilized with 0.1 N acidified CH_3Cl-HCl. The 96-well plate was read at a wavelength of 590 nm on an iEMS Labsystems plate reader. Cell death was confirmed as apoptotic by annexin V staining followed by FACS analysis as described previously (Wen *et al.*, 1997).

IgGκ-NF-κB luciferase reporter gene construct and transfection

IgGκ-NF-κB luciferase reporter gene construct was provided by Peter Kao, Stanford University and have been described previously (Lee *et al.*, 1997). It was stably transfected into A549 and NCI-H1299 cells using lipofectamine-plus (Gibco-BRL, Gaithersburg, MD) and resistant clones were pooled after selection in 400-600 μg/ml of G418.

Luciferase assay

Cells were left untreated or pretreated with triptolide (20 ng/ml) for 5 h and then 9 h after addition of TRAIL (100 ng/ml) cells were harvested for analysis of luciferase activity according to the manufacturer's protocol (Promega Madison, WI). The cells were pretreated with MG132 (3 μM) for 1 h followed by TRAIL treatment for 9 h. Luciferase activity was measured in samples that contained equal protein concentration with a Luminometer (Analytical Luminescence Laboratory, San Diego, CA).

Electrophoretic mobility shift assay (EMSA)

To look at the effect of TRAIL (100 ng/ml) on NF-κB activation, A549 cells were treated as following conditions: (1) unstimulated; (2) TRAIL for 1 h; (3) TRAIL for 2 h; (4) TRAIL for 4 h. For the study of triptolide effect on TRAIL-induced NF-κB activation, A549 cells and NCI-H1299 cells were treated as followings: (1) unstimulated; (2) triptolide (20 ng/ml) for 7 h; (3) TRAIL (100 ng/ml) for 2 h (4) triptolide pretreatment for 5 h followed by TRAIL for 2 h. Nuclear extracts were prepared using a protocol described previously. (Lee *et al.*,1988) Briefly, cells were lysed in buffer A (10 mM Hepes (pH7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2% NP-40 with protease inhibitors). Cell membranes were sheared by 5-6 passages through a 25 gauge needle. Nuclei were pelleted at 12,000 *g* for 15 min, then disrupted in 1 ml buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA plus protease inhibitors) by stirring for 30

min on ice. Chromatin was pelleted at 12,000 *g* and the nuclear extract was dialyzed into buffer D (20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT)) at 4°C for 6 h. Protein concentrations were measured with a Bradford assay (BioRad, Hercules, CA). The EMSA was performed as described previously using a Klenow labeled ^{32}P-IgG NF-κB site as a probe (Rosen *et al.*, 1994). Supershift studies were done with a p65 mAb (Santa Cruz Biochemical, Santa Cruz, CA).

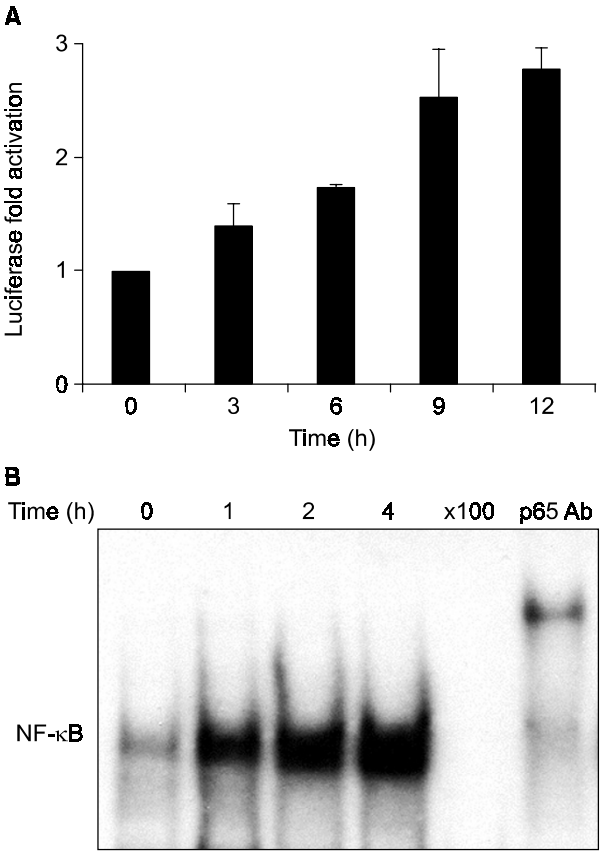

Figure 1. TRAIL induces NF-κB activation in A549 cells. (A) Luciferase assay: Stable A549 IgGκ-NF-κB luciferase cells were treated with TRAIL (100 ng/ml) for different time points. Cells were harvested for luciferase assay. The fold luciferase activation was calculated relative to a normalized value of one given to control (untreated) cells. Data represent the mean luciferase value from triplicates in one expeiment, which was used to calculate the mean ± S.D. from two experiments. (B) EMSA with supershift. A549 cells were treated with TRAIL (100 ng/ml) for various times and harvested for nuclear extraction. Electromobility shift assay was done with a radiolabeled IgGκ-NF-κB probe. Equal amounts (7.5 μg) of nuclear protein was loaded in each lane. In 5th *lane*, an 100× excess of unlabeled oligonucleotide was added 5 min before the addition of radiolabeled probe. In 6th *lane*, a rabbit polyclonal antibody p65 was added to the nuclear extract 10 min before the addition of radiolabeled probe.

Results

TRAIL activates NF-κB in lung cancer cells

EMSA and luciferase reporter gene assays were used to investigate whether TRAIL can activate NF-κB in lung cancer cells. As shown in Figure 1A, enhanced NF-κB binding activity was found 1 h after treatment of A549 cells with TRAIL (100 ng/ml). Activation of NF-κB was further enhanced after 2 h and 4 h, respectively. Antibody reacting to p65 (Rel A) that plays a major role in NF-κB transactivation displayed supershift of the complex demonstrating that p65 is part of the NF-κB complex induced by TRAIL. A similar increase of NF-κB binding activity was also observed in NCI-H1299 cells (data not shown).

TRAIL activation of NF-κB dependent transcriptional activity was examined by luciferase reporter gene assay in the stably transfected cell line with IgGκ-NF-κB luciferase reporter gene construct. TRAIL induced NF-κB activation in A549 cells was increased by two and half fold at 9 h after TRAIL treatment (Figure 3). NF-κB activity induced by TRAIL was about 50% of TNF-α-induced NF-κB activity but TRAIL induced more prolonged NF-κB activity showing progressively increasing tendency up to 12 h. This prolonged TRAIL-induced NF-κB activity might be due to the lack of turn-off by the rebound of IκBα synthesis which itself is a downstream trascript of NF-κB. TRAIL-induced IκBα degradation was observed by western blot analysis but there was no reappearing of IκBα as observed in TNF-induced NF-κB activation (data not shown).

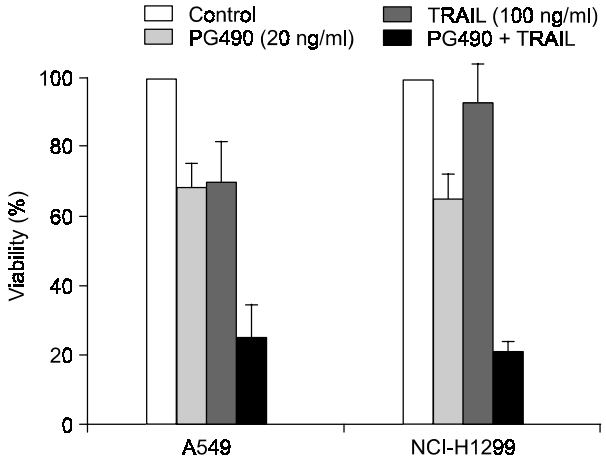

Figure 2. Effect of triptolide and TRAIL on tumor cell viability in A549 and NCI-H1299 lung cancer cell lines. Cell lines were incubated with triptolide and/or TRAIL for 24 h followed by analysis of cell viability by an MTT assay. Values are mean of three experiments ± S.D.

Triptolide sensitizes lung cancer cells to TRAIL-induced apoptosis

A549 and NCI-H1299 cells were relatively resistant to TRAIL. TRAIL alone treatment (100 ng/ml) for 24 h decreased cell viability by 20-30% (Figure 2). Despite increasing dose levels up to 10 fold (1 μM), the viability still remained 50-60%. PG490 alone (20 ng/ml) showed 30% cell death. It was already proven triptolide-induced apoptosis in various tumor cell line. Cell death was confirmed as apoptotic state by annexin V staining followed by FACS anaysis. Pretreatment with triptolide (20 ng/ml) for 5 h followed by the addition of TRAIL 100 ng/ml greatly sensitized both cell lines to TRAIL-induced cell death up to 80% (Figure 2). Triptolide significantly enhanced TRAIL-induced cell death in A549 and NCI-H1299 cells showing a synergistic cytotoxic effect.

Triptolide blocks TRAIL-induced NF-κB transcriptional activity

Recent study showed that triptolide cooperates with TNF to induce apoptosis in solid tumor cells by inhibiting TNF-induced activation of NF-κB (Lee *et al.*, 1999). We tested if triptolide also blocks TRAIL-induced NF-κB activation in lung cancer cells. A549 and NCI-H1299 cells which stably expressed IgGκ-NF-κB luciferase construct were pretreated with triptolide for 5 h followed by the addition of TRAIL. Triptolide alone did not affect basal NF-κB activity but it inhibited TRAIL-induced NF-κB activity almost to the basal level (Figure 3) suggesting that triptolide did sensitize lung cancer cells to TRAIL-induced apoptosis by inhibiting NF-κB activation.

MG132 sensitizes lung cancer cells to TRAIL-induced apoptosis

In addition, another known NF-κB inhibitor, proteasome inhibitor MG132 (N-cbz-Leu-Leu-leucinal) was employed for further confirmation of hypothesis that inhibition of NF-κB sensitizes lung cancer cells to TRAIL-induced apoptosis. Upon exposure to NF-κB activating stimuli, such as TNF-α, IκBα is phosphorylated and targeted for degradation via ubiquitin- proteasome pathway (Palombella *et al.*, 1994). MG132 blocks NF-κB activation by inhibiting proteasomal degradation of IκBα. At the concentration of 3 μM, MG132 alone induced 20-30% of cell death for 24 hr and pretreatment of MG132 1 h before adding TRAIL greatly enhanced TRAIL-induced cell death up to 80-90% in both cell lines (Figure 4). However, this effect was markedly blunted to 50-60% of cell death when MG132 was treated 1 h later after TRAIL addition (data not shown). These results suggest that inhibiting induced NF-κB activity by TRAIL is a major mechanism of enhanced TRAIL-induced apoptosis.

Triptolide does not inhibit the TRAIL-mediated induction of NF-κB DNA binding activity

To determine if triptolide inhibits activation of NF-κB through the inhibition of DNA binding of NF-κB we examined the effect of triptolide on TRAIL-induced binding of NF-κB by EMSA in A549 and NCI-H1299

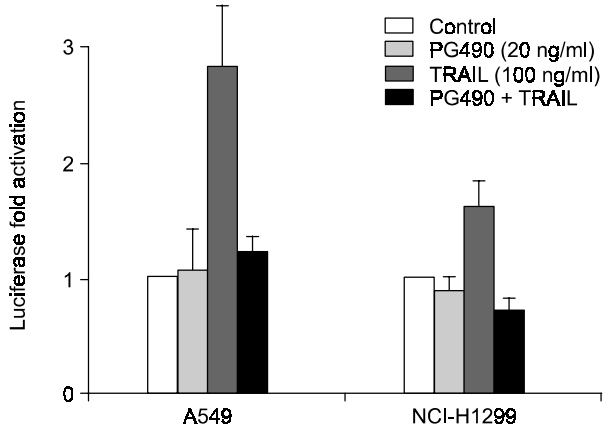

Figure 3. Triptolide blocks TRAIL induction of NF-κB transcriptional activity in A549 and NCI-H1299 lung cancer cells. A549 and NCI- H1299 cells which stably expressed an IgGκ-NF-κB luciferase reporter gene construct were pretreated with triptolide (20 ng/ml) for 5 h followed by the addition of TRAIL (100 ng/ml) for 9 h. The cells were then harvested for analysis of luciferase activity. Values are mean of three experiments ± S.D.

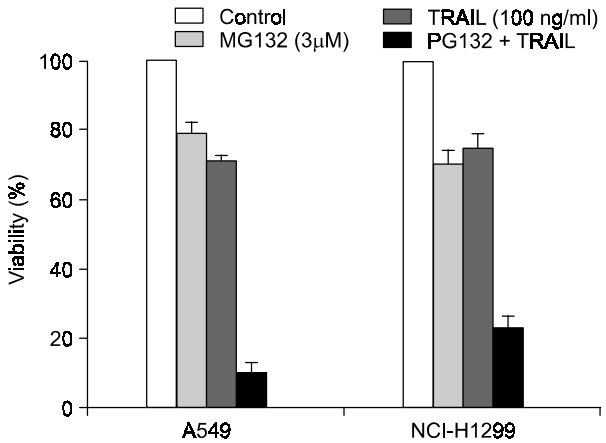

Figure 4. Proteasomal inhibitor MG132 which inhibits IκBα degaradation sensitizes A549 and NCI-H1299 cells to TRAIL-induced apoptosis. Cell lines were pretreated with MG132 (3 μM) and followed with the treatment of TRAIL (100 ng/ml). MTT assay was done for analysis of cell viability. Values are mean of three experiments ± S.D.

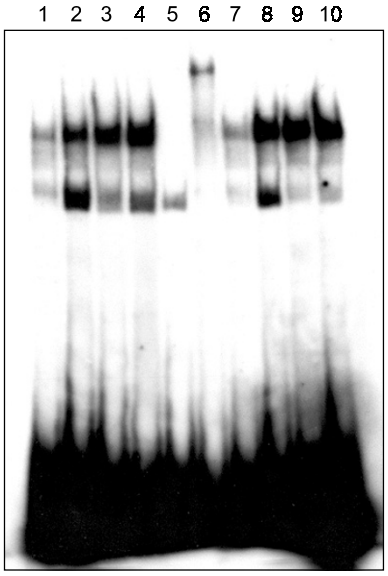

Figure 5. Triptolide does not inhibit the TRAIL-mediated induction of NF-κB DNA binding activity. A549 cells were treated as followings; (1) unstimulated; (2) TRAIL (100 ng/ml) for 1 h, (3) TRAIL (100 ng/ml) for 2 h, (4) TRAIL (100 ng/ml) for 4 h, (5) 100× excess of unlabeled oligonucleotide (6) rabbit polyclonal antibody to p65 (7) triptolide (20 ng/ml) for 1 h; (8) triptolide pretreatment for 5 h followed by TRAIL for 1 h, (9) triptolide pretreatment for 5 h followed by TRAIL for 2 h, (10) Triptolide pretreatment for 5 h followed by TRAIL for 4 h. Nuclear extracts were prepared and analyzed by EMSA in A549 cells with a radiolabelled IgGκ-NF-κB probe. In *lane* 6, a rabbit polyclonal antibody p65 was added to the nuclear extract 10 min before the addition of radiolabeled probe. In *lane* 5, an 100× excess of unlabeled oligonucleotide was added 5 min before the addition of radiolabeled probe.

cells. In Figure 1A, we already showed increased NF-κB DNA binding activity by TRAIL and demonstrated that p65 is part of NF-κB complex induced by TRAIL as evidenced by supershift assay with an antibody to p65 (Rel A). Triptolide did not affect the intensity of the NF-κB complex induced by TRAIL or its migration in A549 cells (Figure 5). Tritolide alone did not induce binding of NF-κB. Antibodies to p65 also supershifted a specific complex in triptolide plus TRAIL-treated cells (Figure 5). Triptolide also did not affect TRAIL-induced binding of NF-κB in NCI-H1299 cells. Recent study demonstrated that triptolide blocks transactivation of p65 through co-transfection of the plasmid encoding fusion protein of the transactivating domains of p65, Gal4-p65$^{521-551}$ (Gal4-p65 TA1) or Gal4-p65$^{268-551}$ (Gal4-p65 TA2), the plasmid encoding DNA-binding domain of the yeast transcription factor Gal4, and a luciferase reporter containing upstream Gal4-binding sites (Lee *et al.*, 1999). Our results confirm that triptolide inhibits transactivation but not DNA binding of NF-κB.

Discussion

The ideal anti-cancer drugs should have two arms, potent tumor cell cytotoxicity and selective tumoricidal effect without adverse effects on normal cells. In this aspect TRAIL has great advantages over preexisting anti-cancer drugs. At least 50% of tumor cell lines tested so far seem to be sensitive to TRAIL-induced apoptosis including lung cancer cells (Griffith *et al.*, 1998). On the other hand, TRAIL did not effect normal, non-transformed mammary epithelial cells, fibroblasts, renal tubular epithelial cells, skeletal smooth muscle cells, pulmonary epithelial cells and melanocytes and there was no detectable adverse effects on mouse viability, tissue integrity, or blood cell counts in *in vivo* SCID mice study (Walczak *et al.*, 1999). In addition, it is cytotoxic to tumor cells regardless of p53 phenotype. These properties provide potential therapeutic value for clinical usefulness of TRAIL in lung cancer frequently harboring p53 mutation.

However, the clinical application of TRAIL might be limited if TRAIL can induce NF-κB activation like TNF. Death inducing ligands such as Fas ligand (FasL/CD95L) and TNF are known to activate NF-κB independently of their cytotoxic function. Here we demonstrated that TRAIL also activates NF-κB in lung cancer cells. Cell specificity of TRAIL-mediated NF-κB activation has been described in some cells but not all solid tumor cell lines (Pan *et al.*, 1997; Sheridan *et al.*, 1997). Jeremias *et al.* (1998) demonstrated that activation of NF-κB by TRAIL was not found in all lymphoid cell lines that are sensitive for TRAIL-induced apoptosis. Even though 'decoy hypothesis' was attractive to the explanation for TRAIL resistance, recent papers indicated no correlation between decoy receptor expression and TRAIL resistance (Griffith *et al.*, 1998; Griffith and Lynch, 1998; Keane *et al.*, 1999).

Several studies have now demonstrated an essential role for NF-κB as an anti-apoptotic transcriptional factor induced by TNF-α, radiation, and some of chemotherapeutic agents (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996). Anti-NF-κB strategy, therefore, seems to be very attractive candidate as a new therapeutic modality for cancer. In this study, we demonstrated anti-NF-κB strategy greatly enhances TRAIL-induced lung cancer cell death using triptolide and MG132. Triptolide is a diterpenoid epoxide extracted from the Chinese herb *Tripterygium wilfordii* that has been used as an immunosuppressant agent in China especially for the treatment of rheumatoid arthiritis (Tao *et al.*, 1995). Previous studies have shown that triptolide itself possesses anti-cancer activity (Kupchan *et al.*, 1972; Shamon *et al.*, 1997). We found that triptolide almost completely blocked TNF-induced NF-κB activation by

inhibiting p65 transactivation but not DNA binding (Lee *et al.*, 1999). Also triptolide was reported to inhibit cytokine production in T cells through inhibition of NF-κB (Qiu *et al.*, 1999). However, the exact mechanism of how triptolide inhibit transactivation of p65 is still to be elucidated. Triptolide might work in the process of p65 phosphorylation, based on recent reports that phosphorylation of p65 is important for transcriptional activation of NF-κB (Wang *et al.*, 1998; Zhong *et al.*, 1998).

We confirmed anti-NF-κB activity for the mechanism by which triptolide sensitized TRAIL-induced apoptosis using another approach to block NF-κB activation. MG132 is known to block IκBα degradation by proteasome. We used low concentration of MG132 (3 μM) because proteasomal inhibitor itself can induce cell death (Shinhara *et al.*, 1996; Gazos Lopez *et al.*, 1997). When pretreatment of MG132 was switched to later time point after addition of TRAIL, the synergy effect of MG132 to TRAIL-induced cell death was markedly blunted suggesting that preceding NF-κB activation plays a key role in resistance to TRAIL.

The mechanism of how inhibition of NF-κB activation sensitizes lung cancer cells to TRAIL-induced apoptosis remains to be elucidated. Because NF-κB is a transcriptional factor, NF-κB dependent anti-apoptotic gene induction should have provided added protection of lung cancer cells from TRAIL-induced apoptosis. A recent study demonstrated that downstream effectors of NF-κB activation which include TRAF-1 (TNFR-associated factor-1), TRAF2, and members of the inhibitor-of-apoptosis (IAP) family, c-IAP1 and c-IAP2, were required to suppress TNF-induced apoptosis (Wang *et al.*, 1998). We proved that triptolide blocked the induction of c-IAP1 and c-IAP2 by TNF-α. However, we failed to demonstrate significant induction of c-IAP1 and c-IAP2 by TRAIL in both A549 and NCI-H1299 cell lines. The difference in the potency of inducing NF-κB between TNF-α and TRAIL might be the reason for this finding. We observed significant differences of TRAIL-induced NF-κB activation from TNF. The potency of TRAIL- induced NF-κB was about half of that induced by TNF but the activity was prolonged. We found relevant evidence for this slow and prolonged NF-κB activation by TRAIL. There was no induction of IκBα by TRAIL-induced activation while turn-off mechanism by this IκBα induction is prominent in TNF-induced NF-κB activation. But it is still unknown that these difference is the reason for the absence of c-IAP1 and c-IAP2 induction by TRAIL.

Acknowledgements

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