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RNAi silencing of the MEKK3 gene promotes TRAIL-induced apoptosis in MCF-7 cells and suppresses the transcriptional activity of NF-κB

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Abstract. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family of cytokines, which can induce apoptotic cell death in a variety of tumor cells or transformed cells, yet, it is relatively non-toxic to most normal cells. Consequently, TRAIL was thought to be a promising agent for cancer therapy. However, recent research reports revealed that many tumors are unresponsive to TRAIL treatment. Apoptotic agents were identified that when used in combination with TRAIL can sensitize tumor cells to TRAIL-mediated apoptosis. It was demonstrated that MEKK3-siRNA sensitized MCF-7 cells to TRAIL cytoxicity. In addition, we investigated the discrepancy of the expression of MEKK3 in breast cancers. It was concluded that elevated MEKK3 expression is found at high frequencies in breast cancer compared to normal breast tissue. Further experiments on the signal machinery showed that MEKK3-siRNA increased the sensitivity of MCF-7 cells to TRAIL by suppressing the transcription activity of NF-κB, and enhancing the caspase-processing to generate executive apoptotic signals. These findings indicate that down-regulation of MEKK3 by siRNA approaches will lead to successful treatment of human breast cancer with TRAIL.

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a novel type of apoptosis inducing ligand. TRAIL has been shown to induce apoptosis selectively in a variety of cancer cell lines but not in normal cells (1,2). Induction of apoptosis by TRAIL is mediated by its interaction with two death domain-containing receptors, TRAIL-R1/DR_4 and TRAIL-R2/DR_5. This, in turn, orchestrates the assembly of the death-inducing signaling complex (DISC) that contains FADD, an adaptor that activates initiator caspases, caspase-8 and caspase-10, leading to direct activation of effector caspases, such as caspase-3 (3,4), or to Bid cleavage and subsequent mitochondrial apoptosis (5,6). Unfortunately, as with a multitude of other chemotherapeutic compounds, TRAIL responsive tumors acquire a resistant phenotype, which renders TRAIL therapy ineffective (7,8). It was reported that TRAIL receptor DR_4 and DR_5 not only transduced apoptotic signals from FADD to caspase family (3), but also activated nuclear factor-kappa B (NF-κB). It could prevent cells from undergoing apoptosis most probably by up-regulating the expression of genes, whose products were critical for suppression of apoptosis (9).

The realization that many tumors are unresponsive to TRAIL treatment has raised interest in identifying apoptotic agents that when used in combination with TRAIL can sensitize tumor cells to TRAIL-mediated apoptosis. Indeed, multiple studies have demonstrated that a variety of apoptotic agents and proteins sensitize several classes of tumor cells to TRAIL-induced apoptosis (10,11). The caspases function to cleave cellular proteins critical for life, including poly (ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, and lamin B.

The role of mitogen-activated protein kinases (MAPKs) in the growth and survival of many cell types has been well documented (12). MAPKs function as mediators that regulate the cellular responses to environmental stress. They serve as key integration points along the signal transduction cascade that not only link diverse extracellular stimuli to subsequent signaling molecules but also amplify the initiating signals to ultimately activate the effector molecules and induce cell proliferation, differentiation, and survival (12,13). The MAPK pathway participates in determining whether a cell undergoes proliferation or cell cycle arrest, or apoptosis (14). Sarker *et al* (15) reported that MAPK plays a crucial role in the regulation of TRAIL-mediated apoptosis in breast tumor MCF-7 cells. The mitogen-activated protein/extracellular signal-regulated kinase kinase kinases (MEKKs) are upstream regulators of the cascade of MAPKs (16).

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MEKK3 is a member of the MEKK subgroup of the MAP3K family (13) and is capable of activating multiple downstream MAPKs, including ERK1/2, JNK, big MAPK/ERK5, and p38 (17-19). In addition, MEKK3 has also been shown to activate the NF-κB pathway (18). However, except for MEKK3, genetic studies have raised uncertainties regarding their involvement in the activation of NF-κB (20,21). Furthermore, studies have shown that overexpression of MEKK3 activates the NF-κB pathways (21,22). Recent studies showed that dysregulation of MEKK3 can affect NF-κB activation (23). In contrast, mouse embryonic fibroblasts deficient in MEKK3 were unable to activate NF-κB in response to stimulation by IL-1 and TNF, indicating the functional importance of MEKK3 in the proinflammatory cytokine-mediated activation of NF-κB (24). Although recently studies showed that cancer cell over-expression of MEKK3 dramatically increased resistance to apoptosis induced by TRAIL, there has been no report that down-regulation of MEKK3 expression could sensitize cancer cell to TRAIL cytotoxicity.

In the present report, we examined whether there is altered expression of MEKK3 in human breast tissue or breast cancer tissue. In addition, we investigated whether down-regulation of MEKK3 was sufficient to lead to lower NF-κB activity and to confer decreased resistance to TRAIL-mediated apoptosis. We report on the role of MEKK3 in protecting MCF-7 cells from TRAIL induced apoptosis.

Materials and methods

Chemicals and reagents. Recombinant human TRAIL was purchased from PeproTech (Rocky Hill, NJ); Anti-MEKK3 monoclonal antibodies were purchased from BD Transduction Laboratories. PARP and β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG complex were provided by Sigma Co. Monoclonal antibodies against NF-κB p65 were purchased from Santa Cruz Biotechnology.

Cell culture and cancer samples. The MCF-7 breast cancer cells were kindly provided by the Department of General Surgery of Renji Hospital (Shanghai Jiaotong University School of Medicine, China), and maintained and cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS, Hyclone) and 100 U/ml penicillin G, 100 mg/ml streptomycin, and 1 mM L-glutamine at 37˚C in a humidified 5% CO_2. The frozen breast tumors, and their matched normal tissues, were obtained from our labortary tumor tissue banks.

Determination of cell proliferation by MTT assay. Equal numbers of cells (~5,000/well) were seeded into a 96-well plate 24 h prior to experimentation. Cells were treated with TRAIL and MEKK3-siRNA alone or in combination. After treatment, MTT dye solution (according to the standard protocol provided by Sigma) was added into the 96-well plate. The corrected absorbance of each sample was calculated by comparison with the untreated control.

Down-regulation of MEKK3 by siRNA. According to data sheets provided by the manufacturer, the scrambled siRNA (proprietary sequence, Dharmacon catalog no. M-003301-02-0005) does not affect gene expression, as determined by gene array experiments. SMARTpool MEKK3 siRNAs were as follows: i) 5'-GAU AGA AGC UCA AGC AUG AUU-3'; ii) 5'-AAA CUC AGC UUU AUG ACA AUU-3'; iii) 5'-CCA AGC AGG UCC AAU UUG AUU-3'; and iv) 5'-GCA AAC GCC UGC AGA CGA UUU-3'. The cells in 35-mm dishes were transfected with the SMARTpool siRNA using Dharma-FECT1 (Dharmacon, Chicago, IL) and incubated for 48 h followed by lysing the cells with lysis buffer. Down-regulation of MEKK3 by siRNA in the cell lysate was confirmed by Western blot assay. Apoptosis of the cells was detected by Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions and followed by flow cytometry.

Western blotting. For preparation of cell extracts, the 48-h transfected cells were washed thrice with ice-cold PBS, and lysed in lysis buffer (Tris-HCl 50 mM pH 8.0, NaCl 150 mM, EDTA 1 mM, Triton X-100 1%, PMSF 100 μg/ml) on ice for 20 min. After centrifuging at 16000 x g for 2 min at 4˚C, the supernatants were analyzed by 10% SDS-PAGE and then electrophoretically transferred to nitrocellulose membrane. After blocking 1 h with 5% fat free milk at room temperature, the membranes were incubated with either anti-MEKK3 monoclonal antibody or anti-β-actin monoclonal antibody, and then reacted with HRP-conjugated secondary antibody. Protein bands were visualized with enhanced chemiluminescence reagent (Pierce, Rockford, IL).

Analysis of PARP cleavage. Cells were harvested in cold PBS and the pellet resuspended in lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Triton X-100) for 20 min at 4˚C. After this incubation, the lysate was centrifuged at 14,000 x g for 20 min at 4˚C to separate membrane (pellet) and cytosolic (supernatant) fractions. Proteins in the supernatant were resolved in 7.5% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes. The blots were then probed with an anti-PARP antibody that detects both the 116-kDa full-length and the 85-kDa cleaved PARP.

Apoptosis detection. The cells after treatment were stained using Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions (BD Biosciences PharMingen, CA, USA). Briefly, cells were washed once with ice cold phosphate-buffered saline (PBS, pH 7.4) and re-suspended in 100 μl binding buffer. The cells were incubated with Annexin V-FITC for 30 min, then propidium iodide (PI) for 5 min at room temperature and added 300 μl binding buffer eventually. The apoptotic cells were analysed with flow cytometry (FACS Calibur, Becton-Dickinson). NE-PER Nuclear and Cytoplasmic Extraction Reagents and LightShift Chemiluminescent EMSA kit were purchased from Pierce.

Electrophoretic mobility shift assay (EMSA). Nuclear proteins were prepared by treating cells with lysis buffer (Pierce) according to the manufacturer's protocol. NF-κB-binding activity was assayed by using electrophoretic mobility shift assay kit (Pierce). A double-stranded oligonuleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3'), corresponding to the putative NF-κB-binding domain was synthesized and end

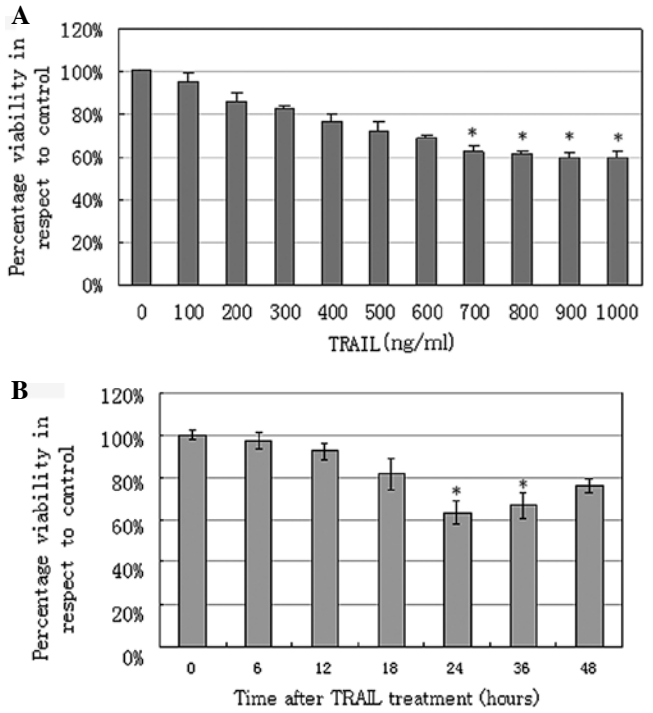

Figure 1. TRAIL reduces cellular viability of the human breast cancer cell line MCF-7. (A) Concentration-response curve for MCF-7 cells with TRAIL (100-1,000 ng/ml) added for 24 h. Cell viability was measured by the MTT assay, and the data presented as percentage in respect to control. Standard deviation of the mean is shown from a minimum of three experiments performed, each consisting of five replicates. *P<0.05 with respect to control (vehicle). (B) Time-course (in hours) measuring cell viability in response to TRAIL (700 ng/m) in MCF-7 cells. Cell viability was measured by the MTT assay, and the data presented as a percentage in respect to control (vehicle). Standard deviation of the mean is shown from a minimum of three experiments performed, each consisting of five replicates. *P<0.05 with respect to control (vehicle).

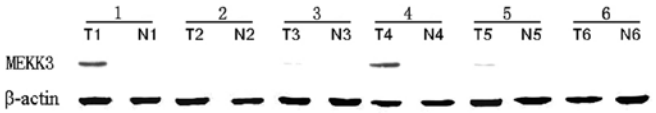

Figure 2. Elevated expression of MEKK3 in breast tumors. Breast tumors (T) and their matched normal (N) breast tissues were homogenized, and the cell extracts were prepared to determine the levels of MEKK3 with anti-MEKK3 antibodies.

labeled with biotin by Shanghai Sangon Company (China). For each reaction, 20 g nuclear proteins were incubated with labeled oligonucleotide at 4˚C for 10 min, then at 25˚C for 20 min. In supershift assays, antibodies specific for the p65 (dilution, 1:1000) or p50 (dilution, 1:1000) subunit of NF-κB were added to the binding system and incubated for 15 min before the addition of the labeled oligonucleotide. Protein-DNA complexes were separated by electrophoresis in 5% non-denaturing polyacrylamide gels, transferred to nylon membranes, and visualized by X-ray film.

Statistical analysis. Results were expressed as the average results of 3 to 6 independent experiments unless indicated otherwise. Differences and correlation between groups were assessed by Mann-Whitney U test. P<0.05 was considered significant.

Results

TRAIL reduces cellular viability of the human breast cancer cell line MCF-7. To determine if TRAIL has potential as an agent against breast cancer, we tested cell viability of the well characterized breast cancer cell line MCF-7 in the presence of TRAIL. To establish the optimal dose of TRAIL, a concentration response (100-1,000 ng/ml) was performed in MCF-7 cells. As demonstrated in Fig. 1A, a decrease in cellular viability reached significant values at 700 ng/ml. Increasing concentrations did not confer a further significant loss in viability. Thus, we chose 700 ng/ml as a suitable concentration to treat with TRAIL.

Furthermore, we determine the optimal exposure time of MCF-7 cells to TRAIL. Fig. 1B shows that a significant decrease in cellular viability occurs after 24 h and this loss continues with time beyond 48 h. TRAIL treatment for 24 h at 700 ng/ml was then chosen for all future experiments.

Elevated MEKK3 levels in breast cancer. It has been reported that elevated MEKK3 expression is found at high frequencies in breast and ovarian cancers. Breast tumors that express high levels of MEKK3 have correspondingly high NF-κB binding activities, whereas tumors with low MEKK3 levels have low constitutive NF-κB binding activities (21). Many types of cancer that have constitutively elevated NF-κB binding activity are resistant to apoptosis by conventional chemotherapies (25,26). To investigate the discrepancy of MEKK3 expression in cancers, we examined the expression of MEKK3 in six human breast tumors and compared them with their matched normal breast tissues from the same patients. When cell extracts from these samples were blotted with anti-MEKK3 antibodies, we observed that although four of the breast tumors have low or undetectable MEKK3, two tumors have significantly elevated MEKK3 (Fig. 2). In contrast, MEKK3 was not detected or detected at very low levels in all six normal matched control tissues.

Inhibition of MEKK3 by siRNA facilitated apoptosis of MCF-7 cells in the presence of TRAIL. To confirm that the expression of MEKK3 was significantly suppressed by MEKK3-siRNA in MCF-7, the MCF-7 cells were transfected with MEKK3-siRNA, and then MEKK3 expression and apoptosis of the cells induced by TRAIL were evaluated. As shown in Fig. 3A, MEKK3 expression was temporarily inhibited by MEKK3-siRNA in MCF-7 cells. We hypothesized that the expression of MEKK3 in MCF-7 cells might attenuate apoptosis induced by TRAIL. To validate this hypothesis, MCF-7 cells were pretreated for 24 h with MEKK3-siRNA followed by treatment with TRAIL at a concentration of 700 ng/ml. Fig. 3B demonstrates that a significant decrease in cellular viability occurs after 48 h. TRAIL combined with MEKK3 remarkably reduced cellular viability to TRAIL. Furthermore, the rate of apoptosis of the transfected MCF-7 cells was remarkably increased to 20-30% (Fig. 3C), indicating again that MEKK3 definitely protects MCF-7 cells from TRAIL cytotoxicity.

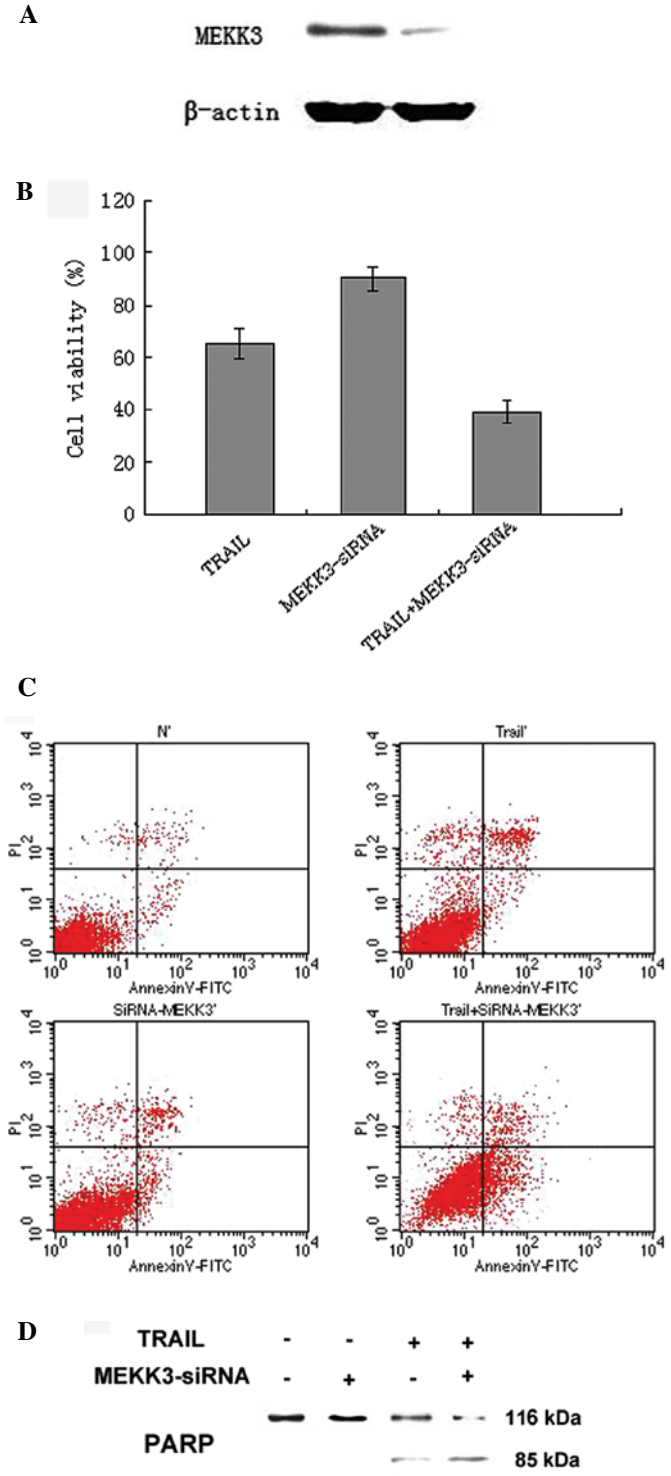

Figure 3. Inhibition of MEKK3 by siRNA increased the sensitivity of MCF-7 cells to TRAIL. MCF-7 cells were transfected with MEKK3-siRNA and incubated. (A) After transfected with MEKK3-siRNA for 48 h, the cells were lysed as described in Materials and methods, then Western blots for MEKK3 was performed. The β-actin was used as the internal control for the equal amount loading of the proteins in the cell lysate. After pretreated with MEKK3-siRNA for 24 h, the cells were treatment with TRAIL for 24 h. (B) Growth curves of MCF-7 cells treated with combination of TRAIL and MEKK3-siRNA. Cell viability was measured by the MTT assay, and the data presented as a percentage in respect to control (vehicle). (C) Apoptosis of the cells was detected by Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions and followed by flow cytometry. (D) Western blot demonstrating the absence of cleaved PARP in MCF-7 cells after the treatment with MEKK3-siRNA for 48 h. The 116-kDa PARP and its 85-kDa cleavage product are indicated. Results are representative of three independent experiments.

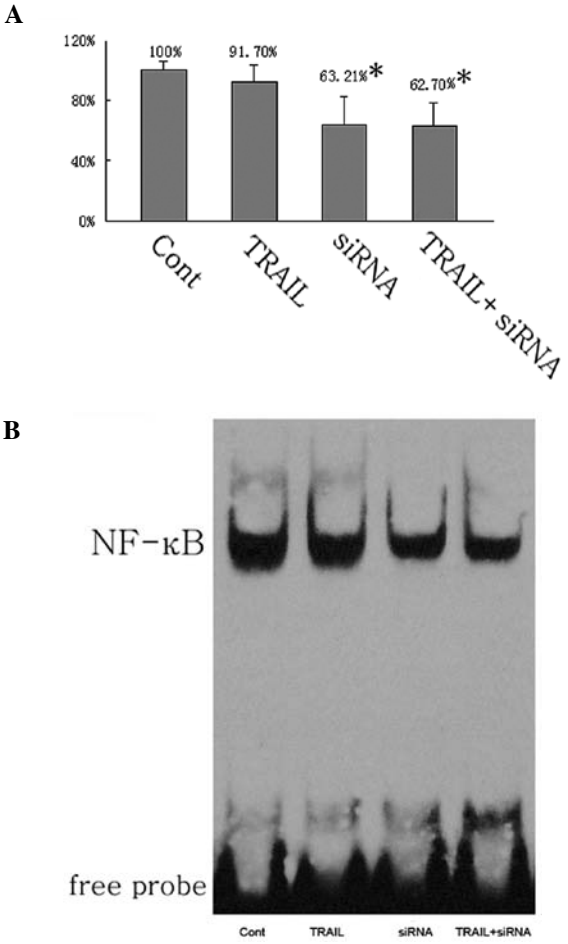

Figure 4. MEKK3-siRNA inhibited TRAIL-induced activation of NF-κB in MCF-7 cells. Nuclear extracts were isolated from MCF-7 cells pretreated with MEKK3-siRNA for 24 h and followed by TRAIL (700 ng/ml) for 24 h as described in Materials and methods. (A) The activation levels of NF-κB in MCF-7 cells were tested by EMSA. Compared with control, the activation levels of NF-κB in MEKK3-siRNA and TRAIL+MEKK3-siRNA groups was significantly suppressed. (*P<0.05, Mann-Whitney U test). (B) The activity of NF-κB was remarkably decreased in MEKK3-siRNA and TRAIL+MEKK3-siRNA groups (representative of 4 independent experiments).

Analysing the cleavage of nuclear poly (ADP-ribose) polymerase (PARP). We also examined the effect of MEKK3-siRNA on TRAIL-induced activation of caspases by analysing the cleavage of nuclear poly (ADP-ribose) polymerase (PARP), a substrate of effector caspases. As shown in Fig. 3D, in TRAIL-treated MCF-7 cells the 85-kDa fragment of PARP was observed. However, generation of the 85-kDa fragment by TRAIL treatment was considerable increased in cells incubated in the presence of MEKK3-siRNA (Fig. 3D), thereby providing further evidence for a positive role of MEKK3-siRNA in the apoptotic signals transduced from TRAIL receptors in MCF-7 cells.

Inhibition of the expression of MEKK3 suppressing the activity of NF-κB. The results of our studies in the MCF-7 cells raise the possibility that the high-expression of MEKK3 in cancer cells may be a potential mechanism that contributes to the resistance of cancer cell. To investigate whether up-regulated expression MEKK3 in MCF-7 cells may, in

part, induce elevated NF-κB activity. In view of the important role of NF-κB in the regulation of apoptosis (27,28), we next investigated the NF-κB activity in the MCF-7 cells treated with MEKK3-siRNA and/or TRAIL. Electrophoretic mobility shift assay (EMSA) further confirmed that NF-κB binding activity with the cis-element was augmented in the cells treated with TRAIL alone or combined with MEKK3-siRNA (Fig. 4). These data suggest that inhibition of MEKK3 sensitizes MCF-7 cells to TRAIL cytotoxicity by suppressing the activity of NF-κB.

Taken together, the above data suggest that the expression of MEKK3 plays an important role in protection of MCF-7 cells from TRAIL-induced cell death. The combination of MEKK3-siRNA and TRAIL suppresses the activity of NF-κB to induce apoptotic signal transduction.

Discussion

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family of cytokines, which can induce apoptotic cell death in a variety of tumor cells, while sparing most normal cells. Ligation of TRAIL with its receptors DR5 and DR4 leads to death-inducing signaling complex (DISC) formation including a rapid association with the adaptor protein Fas-associated death domain (FADD). FADD then conjugates with the initiator caspase-8, leading the classic apoptosis pathways (29).

Plenty of experiments have demonstrated that recombinant TRAIL effectively reduces solid tumor formation and suppresses growth of human cancer cells (30). The underlying mechanism of differential sensitivity of TRAIL in different cell models was initially attributed to cell receptor DR_4 and DR_5. Now more and more research indicates, including breast cancer, that many kind of tumors have to certain extent resistance to TRAIL. There is increasing evidence to demonstrate that the mechanism of TRAIL-resistance might be inside the cells. Activating intracellular anti-apoptosis signal molecules can block the apoptotic signaling pathway. For instance, transcription factor NF-κB could up-regulate many anti-apoptotic genes, such as c-FLIP, XIAP, and cIAP-1, which can inhibit active caspases (31). In the present study, we sought to confirm the effects of MEKK3 in the activation of NF-κB and to seek a molecular explanation for the frequently observed resistance of cancer cells to TRAIL-induced apoptosis. The expression of MEKK3 plays an important role in the up-regulation of NF-κB activity in cancer cells (20,23). This conclusion is further strengthened in this study in which transfection of MEKK3-siRNA resulted in inhibition of NF-κB.

The up-regulation of NF-κB activity has been reported in many cancer cells lines. Intriguingly, we found that a significant portion of breast cancers have elevated expression of MEKK3 with correspondingly high NF-κB activity. In the present study, we have demonstrated that MEKK3 acts as a pro-survival factor in the modulation of apoptosis of MCF-7 cells induced by TRAIL. It is known that MCF-7 cells are relatively resistant to TRAIL cytotoxicity (32). Since the survival signaling pathways may attenuate the cell death induced by TRAIL.

The discrepancy of the expression of MEKK3 was investigated in cancers, we examined the expression of MEKK3 in 6 human breast tumors and compared them with their matched normal breast tissues from the same patients. Two tumors have significantly elevated MEKK3. In contrast, MEKK3 was not detected or detected at very low levels in all 6 normal matched control tissues. Breast tumors that express high levels of MEKK3 have correspondingly high NF-κB binding activities, whereas tumors with low MEKK3 levels have low constitutive NF-κB binding activities, comparable with those of normal control breast tissues. This was consistent with reports by Samanta *et al* (21,22). Many types of cancer that have constitutively elevated NF-κB binding activity are resistant to apoptosis by conventional chemotherapies (25,26).

The experiments demonstrated the sensitivity of MCF-7 cells treated with TRAIL by MTT assay. MCF-7 is relatively resistant to TRAIL. This result agree with the report by Lee *et al* (33). Our experiments demonstrated that MEKK3-siRNA, specifically inhibited the expression of MEKK3, sensitized the cell death induced by TRAIL. Moreover, the cell death of MCF-7 was confirmed by flow cytometry and the increase of cleaved PARP. NF-κB is overexpressed in MCF-7 cells. To further define the effects of MEKK3 in the regulation of NF-κB, cells transfected by MEKK3-siRNA could down-regulate NF-κB.

From these data we may conclude that the apoptosis of MCF-7 cells treated with MEKK3-siRNA and TRAIL is significantly raised, and signals of apoptosis pathway may have important function in MCF-7 apoptosis. To understand molecular mechanism of MEKK3-siRNA sensitized TRAIL-mediated apoptosis in MCF-7 cells, we analyze the biochemical event, which modulates the cell sensitivity to TRAIL. NF-κB is an extensively described anti-apoptotic transcription factor because many of its target genes encode for anti-apoptotic molecules. Constitutive NF-κB activity has been observed in a wide variety of cancer and is associated with resistance to apoptosis (34-36). It has been reported that NF-κB plays a dominant role in TRAIL resistance in breast cancer cell lines (37).

In our experiments TRAIL stimulation did not augment endogenous NF-κB activity, whereas, MEKK3-siRNA suppressed the NF-κB activity significantly in the MCF-7 cells. Combination treatment of MEKK3-siRNA and TRAIL significantly increased the apoptosis of MCF-7. Thus, MEKK3-siRNA dependent inhibition of NF-κB activation might sensitize MCF-7 cells to TRAIL-induced apoptosis. All these results indicate that MEKK3 is responsible for the resistance of MCF-7 cells to TRAIL-induced cell death.

It is noteworthy that stimulation of MEKK3-overexpressed cells with cytokines resulted in dramatic synergistic activation of NF-κB, suggesting functional cooperation between MEKK3 and other signaling molecules activated by the cytokines (21). Indeed, one such molecule downstream of IL-1 and TNF signaling pathway is Akt, which has also been reported to participate in the activation of NF-κB (38,39). Thus, it is likely that MEKK3 and Akt, as downstream targets of IL-1 and TNF, can function cooperatively to regulate NF-κB activation. Other signaling molecules regulating NF-κB activation, however, cannot be excluded. The genetic evidence indicated that MEKK3 is not essential for tumor growth and angiogenesis (40). These data indicate that MEKK3 possesses distinct func-

tions in the regulation of cell proliferation and apoptosis in various cell lines.

In conclusion, it had been reported that NF-κB plays a dominant role in TRAIL resistance in breast cancer cell lines. In this study, we reported that MEKK3-siRNA pretreatment sensitizes MCF-7 cells to TRAIL-induced apoptosis by suppressing the activity of NF-κB. On the basis of the present results, down-regulation MEKK3 by siRNA approaches will lead to successful treatment of human breast cancer with TRAIL.

Acknowledgements

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