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TRAIL induces MMP-9 expression via ERK activation in human astrocytoma cells

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ABSTRACT

Matrix metalloproteinase-9 (MMP-9) is an important angiogenic and prognostic factor in malignant tumors. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is known as the death ligand, which induces preferential apoptosis of transformed tumor cells. In this study, we investigated the biological functions of TRAIL, other than its role in induction of apoptosis. We demonstrated that TRAIL induces MMP-9 expression in human astrocytoma cells, which is preceded by activation of extracellular signal-regulated protein kinase (ERK). In addition, TRAIL induces the DNA-binding activity of NF-κB, an important transcription factor for MMP-9 induction. The specific MEK inhibitor, U0126, significantly blocks TRAIL-mediated NF-κB activation and subsequent MMP-9 induction. These findings indicate that TRAIL treatment in human astrocytoma cells leads to the activation of NF-κB and subsequent expression of MMP-9, which are dependent on ERK activation. Collectively, these results suggest that TRAIL has alternative biological functions in addition to its role in inducing apoptosis in human malignant astrocytoma cells.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily of cytokines. Members of the TNF family contain highly conserved carboxyl-terminal domains and induce receptor trimerization to transduce intracellular signaling [1]. TRAIL can induce apoptotic cell death through caspase-dependent mechanisms [1,2]. TRAIL binds to four different receptors, two of which, DR4 and DR5, induce apoptosis. However, decoy receptors for TRAIL, DcR1 and DcR2, which lack the cytoplasmic death domain for transducing apoptotic death signals, protect cells from TRAIL-induced cell death by interfering with signaling through DR4 and DR5 [2–4]. Transformed tumor cells are generally believed to be more susceptible to TRAIL-mediated cell death due to the selective loss of decoy receptors [5].

Glioblastoma multiforme (GBM) is one of the most aggressive forms of human malignant brain tumors. GBM is characterized by rapid growth, extensive invasiveness and robust neo-angiogenesis. GBM is refractory to all of the current therapeutic approaches including surgery, radiotherapy, and chemotherapy [6]. Locoregional application of TRAIL exerted a strong antitumor activity on intracranial malignant astrocytoma xenografts in athymic mice [7]. In combination with chemotherapeutic agents, TRAIL showed a synergistic cytotoxicity against human astrocytoma or neuroblastoma *in vitro* [8–10]. Therefore, TRAIL is regarded as a promising treatment against resistant malignant GBM.

Matrix metalloproteinases (MMPs) are members of a family of structurally conserved zinc-dependent endopeptidases, which are involved in proteolytic remodeling of the extracellular matrix [11]. The direct correlation between the expression levels of MMPs and the aggressiveness of malignant tumors has been well characterized, both *in vivo* and *in vitro* [12,13]. In particular, gelatinases (MMP-2 and MMP-9) are best known as prognostic factors in many human cancers, including meningioma, breast cancer, glioblastoma, melanoma, gastric cancer, adenocarcinomas, and epithelial cancer [11,14–16]. Inhibition of MMP-9 reduces tumor growth in lung cancer and glioblastoma [17–19]. MMP-9 is involved in the process of tumor-induced angiogenesis by increasing the availability of vascular endothelial cell growth factor, an important angiogenic inducer in malignant tumors [20]. Previously, we demonstrated that the death ligand induces chemokine expression in human astrocytoma cells [21,22]. In this study, we further investigated the alternative biological functions of TRAIL. Our results indicate that treatment with TRAIL significantly increases MMP-9 mRNA, protein expression, and enzymatic activity, and TRAIL-mediated ERK activation involves TRAIL-induced MMP-9 expression.

Materials and methods

Reagents. Human recombinant TRAIL was a generous gift from Dr. Kunhong Kim (Yonsei University, Seoul, Republic of Korea). The MEK inhibitor U0126 and the respective control U0124 were purchased from Calbiochem (San Diego, CA, USA).

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Cell culture. CRT-MG human astrocytoma cells were maintained in RPMI 1640 medium containing 10% FBS (Gibco BRL, Grand Island, NY, USA), 10 mM Hepes (pH 7.2), and 1 mM Earle's balanced salt solution supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, as described previously [21].

Detection of cell death. After treatment with TRAIL, cells were washed twice with phosphate-buffered saline (PBS), trypsinized, suspended in 200 μl binding buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 4% bovine serum albumin (BSA)], and then stained with 0.5 ng of Annexin V-fluorescein isothiocyanate (FITC) and 2.5 ng of propidium iodide (PI) (BD Biosciences Clontech, Palo Alto, CA, USA). Ten thousand cells were analyzed by flow cytometry (FACStar; Becton Dickinson, Mountain View, CA, USA) within 30 min after staining. Cell death, including apoptosis and necrosis, was identified as cell fractions that were stained with Annexin V and/or PI.

RNase protection assay (RPA). Cells were washed with ice-cold PBS, and the RNA was isolated as described previously [23]. Linearized human MMP-9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs were transcribed *in vitro* using T7 RNA polymerase, generating antisense RNA probes. Using 10 μg of total RNA, hybridization was carried out with the MMP-9 and GAPDH riboprobes. Values for mRNA levels were normalized to those of GAPDH mRNA levels under each experimental condition.

Isolation of total RNA and synthesis of cDNA. Cells were washed with ice-cold PBS, and total RNA was extracted using a method based on guanidinium isothiocyanate–phenol extraction followed by ethanol precipitation. To synthesize cDNA, 5 μg RNA was used with the Omniscript RT kit (Qiagen, Valencia, CA, USA). The cDNA produced was stored at −20 °C until needed.

RT-PCR. RT-PCR was performed using the MMP-9 primer set (forward, 5′-CgT CTT CCC CTT CAC TTT CC-3′; reverse, 5′-CAC AgT AgT ggC CgT AgA Ag-3′). PCR conditions were as follows: denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 30 s. The buffer used for PCR was 10 mM Tris–HCl (pH 10), 2.0 mM $MgCl_2$, and 50 mM KCl with 1.25 U of Taq polymerase (Qiagen). After 35 cycles, an additional extension at 72 °C for 10 min was carried out. RT-PCR assays were performed in triplicate.

Western blot. The protein extract was centrifuged to remove cell debris. The total protein concentration in the resulting supernatant was quantified using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Samples of 50 μg protein were then separated using 10% Tris–HCl SDS–PAGE (Bio-Rad Laboratories) and transferred to a nitrocellulose transfer membrane (GE Healthcare, Buckinghamshire, UK). ERK, p-ERK, and MMP-9 were each detected using specific monoclonal antibodies (Cell Signaling Technology, Beverly, MA, USA) diluted 1:500, followed by a rabbit anti-mouse secondary antibody (Cell Signaling Technology). Proteins were visualized using the ECL technique (GE Healthcare).

Gelatin substrate gel zymography. Cells were incubated until they were ~80% confluent; then the medium was aspirated and fresh serum-free medium was added to each dish in the presence and absence of TRAIL. Supernatants (250 μl) were collected after 48 h incubation and concentrated. Concentrated supernatants were mixed with SDS sample buffer without reducing agents, and proteins were subjected to SDS–PAGE in 8% polyacrylamide gels that were copolymerized with 1–2 mg/ml gelatin. After electrophoresis, the gels were washed three times in 2.5% Triton X-100 for 30 min at room temperature to remove the SDS, and then incubated for 24 h at 37 °C in buffer containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$. The gels were stained with Coomassie blue (0.25%) for 30 min, and then destained for 1 h in a solution of acetic acid and methanol. Proteolytic activity was displayed as clear bands (zones of gelatin degradation) against the blue background of the stained gelatin.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts from CRT-MG cells incubated with TRAIL were prepared as described previously [23]. Double-stranded oligonucleotides containing consensus NF-κB sequences (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used in the EMSA. Oligonucleotides were end-labeled with [γ-^{32}P] ATP (Dupont-NEN, Boston, MA, USA) using T4 polynucleotide kinase. Typically, 10 μg of nuclear extracts was equilibrated for 15 min in binding buffer [10 mM Tris–HCl (pH 8.0), 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.25 mM DTT) containing 1 μg of poly dI/dC (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The ^{32}P-labeled oligonucleotide probes (20,000 cpm) were added to the extracts and incubated for an additional 20 min at 4 °C. Bound and unbound probes were then separated by electrophoresis on a 5% native polyacrylamide gel.

Statistical analysis. Data are presented as means ± SD. Levels of significance for comparisons between samples were determined using Student's *t*-test distribution.

Results and discussion

While TRAIL is known to induce apoptotic cell death through caspase-dependent mechanisms, primary astrocytes and transformed astrocytoma cells have been shown to be resistant to TRAIL-mediated cell death, even though they express high levels of death receptors on the surface [21,24]. We hypothesized that TRAIL might have alternative functions in TRAIL-resistant human astrocytoma cells. Previously, we demonstrated that TRAIL-induced pro-inflammatory gene expression, such as interleukin-8 and intercellular adhesion molecule-1 by human astrocytoma cells [21,25].

Among the various prognostic biomarkers of malignant astrocytomas, we analyzed MMP-9 gene expression since it is one of the most important pro-inflammatory and pro-angiogenic factors in human malignant tumors. Gelatin substrate gel zymography analysis demonstrated that TRAIL ligation increased the enzymatic activity of MMP-9, while the same treatment had no effect on the enzymatic activity of MMP-2 in three kinds of human astrocytoma cells (Fig. 1A). RPA analysis showed that TRAIL is a strong inducer of MMP-9 mRNA expression in CRT-MG cells (Fig. 1B). RT-PCR analysis confirmed the finding that TRAIL-induced mRNA expression of MMP-9 in a time-dependent manner (Fig. 1C). Consistent with the mRNA results, TRAIL enhanced MMP-9 protein expression in a time-dependent manner (Fig. 1D). These results clearly indicate that TRAIL ligation induces MMP-9 expression at the mRNA and protein level.

To assess the mechanisms involved in the induction of MMP-9 by TRAIL treatment, we probed the involvement of mitogen-activated protein kinases (MAPKs) in CRT-MG cells with TRAIL treatment over time (0–8 h). Western blot analysis indicated that extracellular signal-regulated protein kinase (ERK) was activated as early as 1–2 h after TRAIL treatment (Fig. 2A). We further investigated whether NF-κB, a well-known transcription factor, is also involved in TRAIL-induced MMP-9 expression in human astrocytoma cells. EMSA results showed clearly that DNA-binding activity of NF-κB increased markedly upon TRAIL treatment in a time-dependent manner (1–3 h) (Fig. 2B). To determine whether TRAIL-mediated ERK activation is necessary for TRAIL-mediated NF-κB activation in human astrocytoma cells, we used a pharmacological MEK inhibitor, U0126. Pretreatment with U0126 for 1 h blocked TRAIL-mediated NF-κB activation in CRT-MG cells, suggesting that activation of ERK is required for TRAIL-mediated NF-κB activation (Fig. 2C).

We examined whether TRAIL-mediated ERK activation is necessary for inducing MMP-9 expression in human astrocytoma cells.

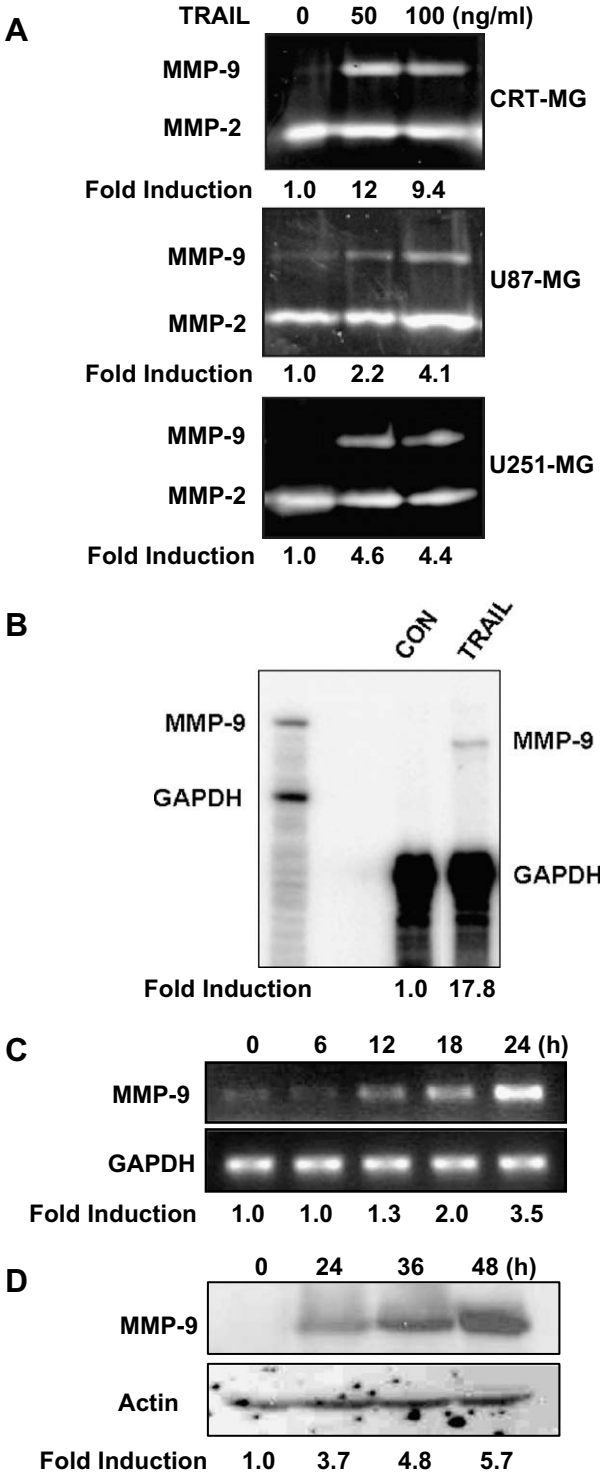

Fig. 1. TRAIL induces MMP-9 expression in human astrocytoma cells. (A) Three kinds of human astrocytoma cells were incubated with TRAIL (0–100 ng/ml) for 48 h, and gelatin gel zymography was performed for analysis of the enzymatic activity of MMP-9. Fold induction of MMP-9 was normalized to the level of untreated control. (B) CRT-MG cells were incubated with TRAIL (50 ng/ml) for 24 h, and total RNA was examined for MMP-9 mRNA expression by RPA. Fold induction of mRNA was normalized to the level of GAPDH mRNA expression. (C) CRT-MG cells were incubated with TRAIL (50 ng/ml) for 0–24 h, and the total RNA was examined for MMP-9 mRNA expression by RT-PCR. Fold induction of mRNA was normalized to the level of GAPDH mRNA expression. (D) CRT-MG cells were incubated with TRAIL (50 ng/ml) for 0–48 h and then immunoblot analysis was performed for measurement of MMP-9 protein expression. Data shown are representative of three independent experiments.

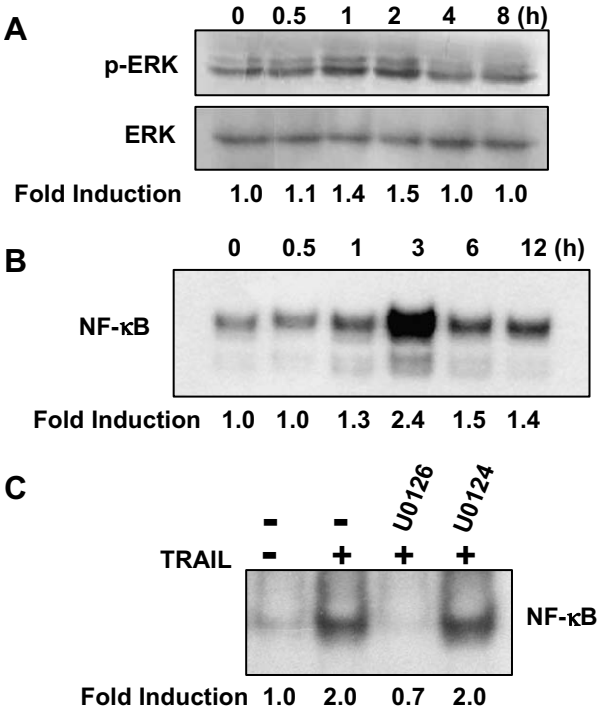

Fig. 2. TRAIL induces activation of ERK and NF-κB in human astrocytoma cells. (A) CRT-MG cells were treated with TRAIL (50 ng/ml) for 0–8 h. Activation of ERK was determined by the degree of phosphorylation as detected by Western blot using antibodies specific for phosphorylated forms of kinases. Total ERK was assessed by internal controls for the amounts of protein loaded. (B) CRT-MG cells were incubated with TRAIL (50 ng/ml) for 0–12 h. Nuclear extracts were analyzed for the nuclear NF-κB binding activity by EMSA using the human consensus NF-κB probe. (C) CRT-MG cells were preincubated with U0126 (10 μM) for 1 h and treated with TRAIL (50 ng/ml) for 3 h; then the nuclear extracts were analyzed for NF-κB binding activity by EMSA using the human consensus NF-κB probe. U0124 (10 μM) was used as the negative chemical control for U0126. Data shown are representative of three independent experiments.

Pretreatment with U0126 for 1 h significantly blocked TRAIL-induced MMP-9 mRNA and protein expression, while preincubation with U0124, a negative chemical control of U0126, had no effect on TRAIL-induced MMP-9 expression (Fig. 3A and B). Blockage of ERK activation also suppressed TRAIL-induced enzyme activity of MMP-9 in a dose-dependent manner in CRT-MG cells (Fig. 3C). Next, we examined whether TRAIL-mediated NF-κB activation is necessary for inducing MMP-9 expression in human astrocytoma cells by using a pharmacological NF-κB inhibitor, MG-132. Pretreatment with MG-132 for 1 h significantly blocked TRAIL-induced MMP-9 mRNA and protein expression (Fig. 4A and B). Blockage of NF-κB activation also completely suppressed TRAIL-induced enzyme activity of MMP-9 in CRT-MG cells (Fig. 4C). Collectively, these results suggest that TRAIL-induced MMP-9 expression requires the activation of ERK and NF-κB.

MMP-9 is an important protease for angiogenesis and invasion in tumor development [26,27]. It is expressed highly in human astrocytoma cells, whereas, MMP-9 is not expressed in normal brain tissue [28]. MAPKs are regarded as important signal mediators for MMP-9 expression in various cell types and stimuli. The phorbol 12-myristate 13-acetate induces MMP-9 expression in human astrocytoma cells through activation of ERK and JNK [27], and interleukin-1β induces MMP-9 expression in human smooth muscle cell via ERK, JNK, and p38 MAPK activation [29]. In this study, we demonstrated that TRAIL activates the ERK pathway, and that inhibition of ERK activation blocks TRAIL-induced MMP-9 expression in human astrocytoma cells.

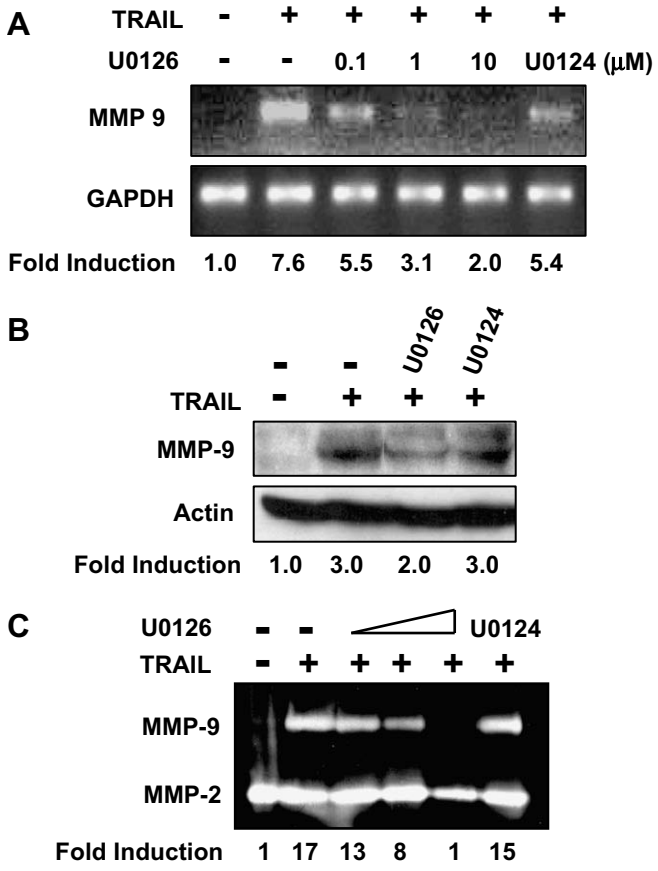

Fig. 3. Inhibition of ERK activation abrogates TRAIL-mediated MMP-9 induction. (A) Expression of MMP-9 mRNA measured by RT-PCR. Samples were taken from CRT-MG cells that were preincubated with three concentrations (0.1, 1, 10 μM) of MEK inhibitor U0126 for 1 h and then treated with TRAIL (50 ng/ml) for 24 h. (B) Expression of MMP-9 protein measured by immunoblot analysis. Samples of CRT-MG cells were first preincubated with U0126 (10 μM) for 1 h and then treated with TRAIL (50 ng/ml) for 48 h. (C) Enzymatic activity of MMP-9 measured by gelatin gel zymography (samples were prepared as described in (A)). CRT-MG cells were treated with TRAIL (50 ng/ml) for 48 h. Data shown are representative of three independent experiments.

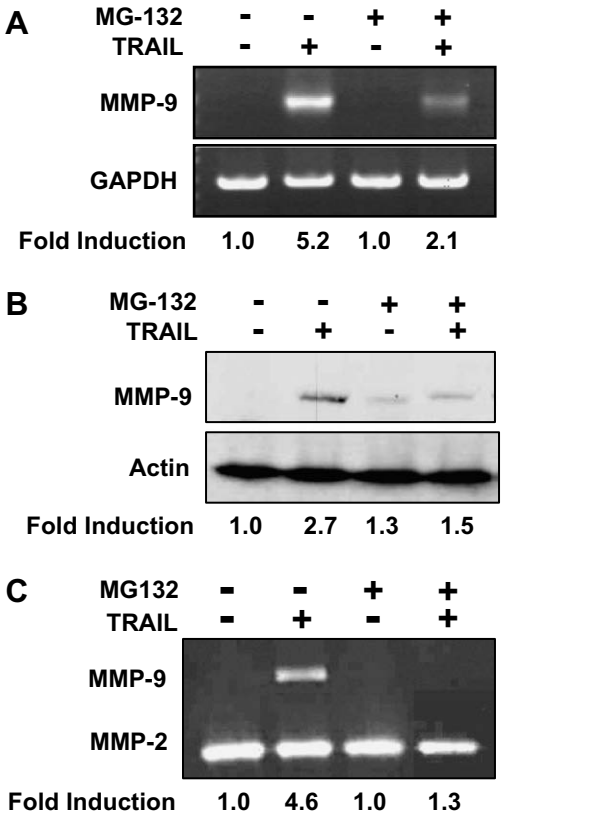

Fig. 4. Inhibition of NF-κB activation abrogates TRAIL-mediated MMP-9 induction. (A) Expression of MMP-9 mRNA measured by RT-PCR. Samples were taken from CRT-MG cells that were preincubated with MG-132 (0.1 μM) for 1 h and then treated with TRAIL (50 ng/ml) for 24 h. (B) Expression of MMP-9 protein measured by immunoblot analysis (samples were prepared as described in (A)). CRT-MG cells were treated with TRAIL (50 ng/ml) for 48 h. (C) Enzymatic activity of MMP-9 measured by gelatin gel zymography (samples were prepared as described in (A)). CRT-MG cells were treated with TRAIL (50 ng/ml) for 48 h. Data shown are representative of three independent experiments.

Regulatory elements within the 5′ flanking sequence of the human MMP-9 gene have consensus sequences for transcriptional factors such as NF-κB, Sp1, Ets, and AP-1 [30]. Activation of NF-κB requires proteosomal degradation of IκB, which is dependent on ubiquitination of IκB [31]. Proteosome-dependent degradation of IκB is also involved in TRAIL-induced NF-κB activation. We showed that inhibition of ERK activation blocks TRAIL-induced activity of NF-κB, suggesting that activation of ERK plays an essential role in TRAIL-mediated NF-κB activation and subsequent MMP-9 expression in human astrocytoma cells. Since the proteosomal inhibitor MG-132 abrogated TRAIL-induced NF-κB activation and subsequent induction of MMP-9 expression, proteosomal degradation of IκB seems to play an important role in TRAIL-induced NF-κB activation and subsequent MMP-9 expression.

In previous work, we observed that caspase inhibition blocks TRAIL-induced IL-8 expression in human astrocytoma cells [21], yet in the present study, caspase inhibition did not affect TRAIL-induced MMP-9 expression in human astrocytoma cells (data not shown). Therefore, further studies are needed to probe the interaction between the death domain of TRAIL and ERK upon intracellular signaling of TRAIL-induced MMP-9 expression. Although MMP-2 and MMP-9 have similar substrate specificities, their expression patterns displayed different responses to TRAIL. Consistent with previous reports, growth factor induced expression of MMP-9, but not that of MMP-2 in bladder cancer and malignant mesothelioma cells [32,33]. Different MMP genes have been postulated to be differentially regulated since MMP genes have diverse promoter elements [34]. We could not observe TRAIL-mediated MMP-9 expression in human neuroblastoma cells. It is indicated that human astrocytoma cells prefer to TRAIL-mediated MMP-9 induction in brain tumor.

The use of TRAIL has been tested as a potential anticancer therapy because it is known to induce apoptosis in various cancer cells, but not in normal cells [1]. Some highly malignant tumors are resistant to TRAIL-mediated apoptosis [35], suggesting the possibility that TRAIL may evoke different biological functions in these tumor cells. In this study, we demonstrated an alternative biological function of TRAIL: induction of MMP-9 expression through the ERK signal pathway in malignant human astrocytoma cells. Therefore, we suggest that treatment with TRAIL, in combination with anti-angiogenesis therapy against MMP-9, may pose a more effective brain cancer therapy. A better understanding of the role of TRAIL may provide the therapeutic strategy for the effective treatment of cancer.

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