The involvement of reactive oxygen species (ROS) and p38mitogen-activated protein (MAP) kinase in TRAIL/Apo2L-induced apoptosis

Myoung-Woo Lee[a], Soon Cheol Park[b], Young Geun Yang[c], Soon Ock Yim[d], Hee Sun Chae[a], Jae-Hyung Bach[a], Hyun Jung Lee[a], Kyung Yong Kim[a], Won Bok Lee[a,*], Sung Su Kim[a,c,*]

[a] *Department of Anatomy, College of Medicine, Chung-Ang University, 221 Huksuk-dong, Dongjak-ku, Seoul 156-756, South Korea*
[b] *Department of Life Science, College of Natural Science, Chung-Ang University, 221 Huksuk-dong, Dongjak-ku, Seoul 156-756, South Korea*
[c] *Biograd Inc., Chung-Ang University, 221 Huksuk-dong, Dongjak-ku, Seoul 156-756, South Korea*
[d] *School of Oriental Medicine, South Baylo University, Anaheim, CA, USA*

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Abstract To determine the apoptotic signaling pathway which tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) induced, we investigated the contribution of reactive oxygen species (ROS), p38 mitogen-activated protein (MAP) kinase and caspases in human adenocarcinoma HeLa cells. Here we show that upon TRAIL/Apo2L exposure there was pronounced ROS accumulation and activation of p38 MAP kinase, and that activation of caspases and apoptosis followed. Pretreatment with antioxidants such as glutathione or estrogen attenuated TRAIL/Apo2L-induced apoptosis through a reduction of ROS generation and diminished p38 MAP kinase and caspase activation. The p38 MAP kinase inhibitor SB203580 prevented apoptosis through a blockage of caspase activation, although ROS generation was not attenuated. Furthermore, the pan-caspase inhibitor *Z*-Val-Ala-DL-Asp-fluoromethyl ketone fully prevented apoptosis, while neither ROS accumulation nor p38 MAP kinase activation were affected. Therefore, our results suggest that TRAIL/Apo2L-induced apoptosis is mediated by ROS-activated p38 MAP kinase followed by caspase activation in HeLa cells. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Tumor necrosis factor-related apoptosis-inducing ligand; Reactive oxygen species; p38 mitogen-activated protein kinase; Caspase; Apoptosis

1. Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo2L) is a member of the TNF family, which includes TNF, FasL, CD27L, OX40, CD30L, and CD40L [1,2]. The TNF family is involved in a variety of cellular processes, including cell proliferation, differentiation and apoptosis [3,4]. TRAIL/Apo2L induces apoptosis by binding to the death receptor (DR)4 (TRAIL-R1) and DR5 (TRAIL-R2) [5–7]. Both DR4 and DR5 contain a conserved cytoplasmic region called 'death domain' that is required for DR4- and DR5-induced apoptosis [6–8]. Binding of TRAIL/Apo2L to DR4 and DR5 results in activation of the caspase cascade, presumably through recruiting FADD or related adaptor molecules [9,10]. However, the intracellular mechanism of TRAIL/Apo2L-induced apoptosis is not fully understood.

Oxidative stress constitutes a major threat to organisms living in an aerobic environment, and for humans it might have a causative role in many disease processes. All aerobic cells generate reactive oxygen species (ROS) including superoxide radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$). ROS have been shown to induce various biological processes, including apoptosis or programmed cell death [11,12]. For example, ROS have been implicated in various cytotoxic events including transforming growth factor β-, ultraviolet radiation (UV)- and 3-hydroxykynurenine-induced cell death [13–15]. In addition, intracellular ROS has been reported to be involved in TNF induced apoptosis [16].

Mitogen activated protein (MAP) kinase signal transduction pathways in mammalian cells include the extracellular signal related kinase (ERK), c-Jun N-terminal kinase (JNK/SAPK) and p38 MAP kinase. ERK is generally associated with proliferation and growth factors. In contrast, JNK and p38 MAP kinase are induced by stress responses and cytokines, and can mediate differentiation and cell death [17–19]. p38 MAP kinase has been distinctively implicated in the regulation of various cellular processes. The activation of p38 MAP kinase has been observed in a number of physiological responses such as apoptosis of myocardial cells [20] and adipogenesis in 3T3-L1 cells [21]. A specific inhibitor of p38 MAP kinase blocks apoptosis induced by ceramide [22] and UV [23]. Moreover, early membrane blebbing during oxidative stress-induced apoptosis is tightly regulated by p38 MAP kinase-mediated actin organization [24]. Although p38 MAP kinase has been reported to be involved in various physiological events, the mechanism of activation of p38 MAP kinase by various stimuli as well as its biological roles remain to be elucidated.

Caspases, a family of aspartate-specific cysteine proteases,

*Corresponding authors. Fax: (82)-2-826 1265.
E-mail addresses: whitefox@cau.ac.kr (W.B. Lee), sungsu@cau.ac.kr (S.S. Kim).

Abbreviations: TRAIL/Apo2L, tumor necrosis factor-related apoptosis-inducing ligand; DR, death receptor; ROS, reactive oxygen species; p38 MAP kinase, p38 mitogen-activated protein kinase; TNF, tumor necrosis factor; MTT, 3-(4,5-dimethyl-thiozol-2-yl)-2,5-diphenyl-tetrazolium bromide; zVAD-fmk, *Z*-Val-Ala-DL-Asp-fluoromethyl ketone; GSH, glutathione

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are the key effectors responsible for many of the morphological and biochemical changes of apoptosis [25] and as such are important in the implementation of apoptosis. The involvement of the caspases in apoptosis has been demonstrated in an increasing number of biological systems [26]. Furthermore, evidence for caspase activation was observed in HL-60 cells following ROS-induced p38 MAP kinase activation [27].

In the present study to explore the intracellular molecular mechanism of TRAIL/Apo2L, we investigated the possibility that ROS, p38 MAP kinase and caspases are implicated in TRAIL/Apo2L-induced apoptosis in HeLa cells. Thereby, we tried to further elucidate the relationship between these factors in TRAIL/Apo2L-induced apoptosis. Our results suggest that TRAIL/Apo2L-induced apoptosis is mediated by ROS-activated p38 MAP kinase followed by caspase activation in HeLa cells.

2. Materials and methods

2.1. Cell culture

Human adenocarcinoma HeLa cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Burlinton, ON, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies) in a humidified 95% air, 5% CO_2 incubator. The cells were transferred to low serum media (1% FBS) 2 h before the treatment with recombinant human TRAIL/Apo2L (Biomol, Plymouth, PA, USA).

2.2. Cell viability assay

Cells were plated on 96-well plates (Corning, NY, USA) at a density of 5×10^4 cells/well, in 100 μl of 10% FBS/DMEM without phenol red and incubated for 24 h. 2 h before cell stimulation, cell media was replaced with 90 μl of 1% FBS/DMEM without phenol red. Plates were pretreated 2 h with either vehicle, glutathione (GSH; Sigma, St. Louis, MO, USA), estrogen (17α-estradiol) (Sigma), SB203580 (Calbiochem, San Diego, CA, USA) or *Z*-Val-Ala-DL-Asp-fluoromethyl ketone (zVAD-fmk; Enzyme System Products, Livermore, CA, USA) prior to 4 ng/ml TRAIL/Apo2L treatment. 2 h before the end of the treatment with TRAIL/Apo2L, 10 μl 10% Triton X-100 and then 11 μl of 3-(4,5-dimethyl-thiozol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) 10× solution (10 mg/ml) were added. At termination all media and MTT solutions were suctioned off, cells and crystallized dyes were dissolved by adding 100 μl of 100% dimethylsulfoxide and shaking for 20 min. Absorbance at 570 nm was measured with an enzyme-linked immunosorbent assay (ELISA) Reader (Molecular Devices, Sunnyvale, CA, USA). Following the addition of 10% Triton X-100, assay values were obtained with vehicle treatment taken as 100% and MTT reduction defining complete inhibition (0%).

2.3. Determination of ROS generation

Hydrogen peroxide generation induced by TRAIL/Apo2L was measured by incubation with fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA; Sigma). HeLa cells were stained with 10 μM of DCF-DA for 30 min. Cells were collected and washed twice with phosphate-buffered saline (PBS). Cells were put on slide-glasses and were analyzed on a fluorescent microscope (Olympus IX 70, Tokyo, Japan).

2.4. Western blotting

For Western blot analysis, HeLa cells were washed with PBS, the cells pelleted and then lysed by the addition of cold RIPA buffer (1% Triton X-100, 20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM Na_3VO_4, 40 mM NaF, 5 mM EGTA, 0.2% sodium dodecyl sulfate (SDS), 0.2 mM phenylmethylsulfonyl fluoride, 100 μM leupepsin). The cell lysate was centrifuged at $15\,800\times g$ at 4°C for 10 min. The supernatant was harvested and analyzed for protein content using a protein assay kit (Bio-Rad, Hercules, CA, USA). For electrophoresis, proteins (80 μg) were dissolved in sample buffer (5% β-mercaptoethanol, 15% glycerol, 3% SDS, 0.1 M Tris, pH 6.8), boiled for 3 min, and then separated on a 10% SDS gel under reducing conditions. The separated proteins were transferred onto polyvinylidene difluoride membranes using a semidry trans-blot system (Schleicher and Schuell, Germany). The blots were blocked for 2 h at room temperature with Tris-buffered saline (TBS) (10 mM Tris, pH 7.5, 100 mM NaCl) containing 5% non-fat dry milk. The blots were washed three times with TBS, and then incubated at room temperature overnight with anti-p38 MAP kinase antibodies (1:1000 dilution) (Santa Cruz Biotech, Santa Cruz, CA, USA) or anti-phospho-p38 MAP kinase antibodies (1:1000 dilution) (Santa Cruz Biotech) in TBST (10 mM Tris, pH 7.5, 100 mM NaCl, 0.05% Tween 20) containing 3% non-fat dry milk. The next day the blots were washed three times with TBST, and then incubated for 2 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution) (Santa Cruz Bio-

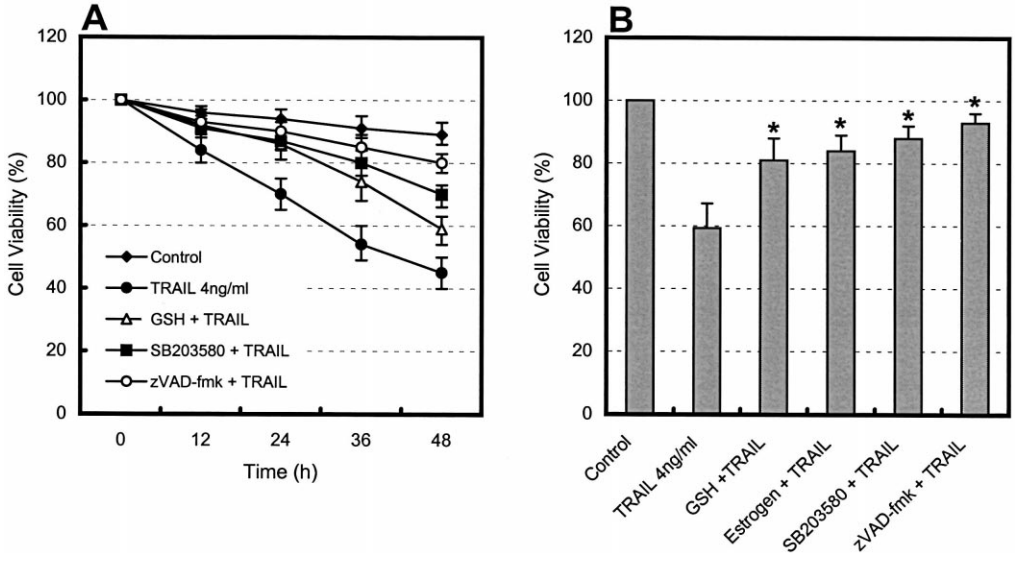

Fig. 1. Effect of GSH, SB203580, or zVAD-fmk on TRAIL/Apo2L-induced cell death. A: HeLa cells were pretreated with 10 μM of GSH, 10 μM of SB203580, or 10 μM of zVAD-fmk for 2 h and then treated with 4 ng/ml of TRAIL/Apo2L. Cell viability was determined at indicated time points. B: A bar graph of cell viability as by the MTT assay at 36 h after 4 ng/ml TRAIL/Apo2L treatment. Values, presented as percentage of control cells incubated with vehicle, are mean ± S.E.M. of four separate experiments. *The difference from the cells treated with TRAIL/Apo2L alone was statistically significant ($P < 0.05$).

tech) in TBST containing 3% non-fat dry milk. After washing three times with TBST, the protein was visualized using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, UK). The relative protein levels were determined using densitometry (Vilber Lourmat, France) normalizing to the p38 MAP kinase band on a duplicate blot.

2.5. Caspase substrate cleavage assay

HeLa cells were collected by centrifugation at $150\times g$ for 10 min, and the washed cell pellet was resuspended in 100 μl of lysis buffer containing 50 mM Tris, pH 7.5, 0.03% NP-40, and 1 mM dithiothreitol. The lysates were incubated on ice for 20 min with intermittent vortex and then centrifuged at $15\,800\times g$ for 5 min. The supernatant was harvested and analyzed for its protein content using a protein assay kit (Bio-Rad). To assess caspase cleavage, 20 μg of cellular extracts were incubated with 0.25 mM zVAD-PNA (Enzyme System Products), a substrate of pan-caspase, in 100 μl of total volume at 37°C for 1 h. Absorbance at 405 nm was measured with an ELISA Reader (Molecular Devices). Enzymatic activity is expressed as arbitrary units of relative value.

2.6. Statistical analysis

Data are expressed as mean ± S.E.M. values. Student *t*-test was applied to study the relationship between the different variables. A $P < 0.05$ was considered to be significant.

3. Results

3.1. Involvement of ROS and p38 MAP kinase in TRAIL/Apo2L-induced apoptosis

To investigate the involvement of ROS in TRAIL/Apo2L-induced apoptosis, a fluorescent analysis with DCF-DA was performed to quantify intracellular levels of ROS. As shown in Fig. 2A,B,G, when cells were treated with 4 ng/ml of TRAIL/Apo2L for 1 h, the intensities of DCF fluorescence showed significant, above five-fold increases, than those in untreated cells. The increase in DCF fluorescence was largely attenuated by pre-incubation of antioxidant such as GSH or estrogen (Fig. 2C,D), indicating that the increased fluorescence reflects increased levels of intracellular ROS. Pre-incubation of the HeLa cells with antioxidants effectively prevented TRAIL/Apo2L-induced apoptosis corroborates (Fig. 1A,B). These results implicate ROS in TRAIL/Apo2L-induced apoptosis in HeLa cells.

To identify the activation of p38 MAP kinase in TRAIL/Apo2L-treated HeLa cells, we used polyclonal antibody that recognizes only the activated, phosphorylated form of p38 MAP kinase. As shown in Fig. 3, TRAIL/Apo2L strongly stimulated p38 MAP kinase activation. In HeLa cells exposed to TRAIL/Apo2L, the p38 MAP kinase activity was increased within 0.5 h, reached the maximum at 2 h, and returned to basal levels at 12 h. Total levels of p38 MAP kinase were not affected by treatment of TRAIL/Apo2L and were therefore used as an internal standard in quantification of p38 MAP kinase activation. To address the contribution of p38 MAP kinase to induce cell death in HeLa cells, we examined the effect of p38 MAP kinase inhibition on TRAIL/Apo2L-induced apoptosis. Concurrent addition of 10 μM of SB203580, a specific inhibitor of p38 MAP kinase, effectively prevented TRAIL/Apo2L-induced apoptosis (Fig. 1A,B) and attenuated p38 MAP kinase activation induced by TRAIL/Apo2L (Fig. 4A,B). These results indicate that TRAIL/Apo2L strongly activates p38 MAP kinase in TRAIL/Apo2L-induced apoptosis, contributing to the potential induction of the apoptotic cascade.

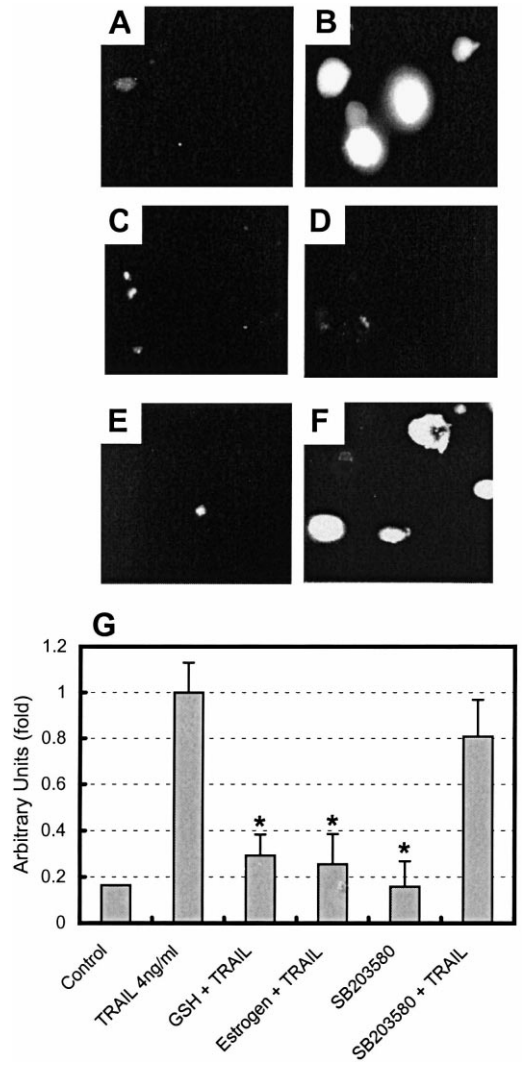

Fig. 2. Determination of ROS generation levels by TRAIL/Apo2L after pretreatment with GSH, estrogen, or SB203580. HeLa cells were untreated (A) or treated (B) with 4 ng/ml of TRAIL/Apo2L for 1 h. Pretreatment of HeLa cells with 10 μM of GSH (C) or 100 nM of estrogen (D) were treated with 4 ng/ml of TRAIL/Apo2L for 1 h. Pretreatment of HeLa cells with 10 μM of SB203580 were either not treated (E) or treated (F) with 4 ng/ml of TRAIL/Apo2L for 1 h. Hydrogen peroxide generation induced by TRAIL/Apo2L was measured by incubation with fluorescent probe 2, DCF-DA. The figures are representative for four different experiments. G: Quantification of fluorescent levels (results of A–F). Fluorescent levels are expressed as arbitrary units of relative value. *The difference from the cells treated with TRAIL/Apo2L alone was statistically significant ($P < 0.05$).

3.2. Activation of p38 MAP kinase by TRAIL/Apo2L-induced ROS

To assess whether p38 MAP kinase activation is induced by ROS, we studied the effect of pretreatment with antioxidants on TRAIL/Apo2L-induced p38 MAP kinase activation. As shown in Fig. 4, the p38 MAP kinase activation induced by TRAIL/Apo2L was effectively inhibited by pretreatment with 10 μM of GSH or 100–500 nM of estrogen. Although, the pretreatment with 10 μM of SB203580 did not attenuate TRAIL/Apo2L-induced ROS generation (Fig. 2F,G). As observed in untreated cells, treatment with SB203580 alone did not affect the ROS generation (Fig. 2E,G). Therefore, these

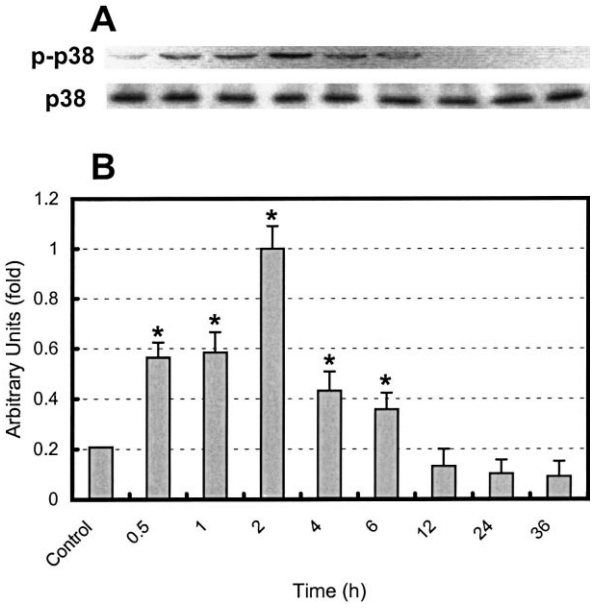

Fig. 3. Western blot analysis of p38 MAP kinase in TRAIL/Apo2L treated HeLa cells. A: HeLa cells were untreated (control; lane 1) or treated with 4 ng/ml of TRAIL/Apo2L for 0.5 (lane 2), 1 (lane 3), 2 (lane 4), 4 (lane 5), 6 (lane 6), 12 (lane 7), 24 (lane 8) and 36 h (lane 9). The phosphorylation state of p38 MAP kinase was detected by Western blotting with a phosphospecific p38 MAP kinase antibody at indicated time points (p-p38). The same samples were probed with p38 MAP kinase antibody as a loading control (p38). B: Quantification of A results. Total levels of p38 MAP kinase were used as an internal standard in quantification of phosphorylated p38 MAP kinase. The levels of p38 MAP kinase are expressed as arbitrary units of relative value. *The difference from the cells incubated with vehicle alone was statistically significant ($P < 0.05$).

results suggest that ROS activates p38 MAP kinase in TRAIL/Apo2L-induced apoptosis.

3.3. Caspase activation by ROS-activated p38 MAP kinase in TRAIL/Apo2L-induced apoptosis

To determine whether caspase activation is functionally important for TRAIL/Apo2L-induced apoptosis, cells were preincubated with the pan-caspase inhibitor zVAD-fmk. The pretreatment with 10 μM of zVAD-fmk significantly prevented cell death following a 36 h exposure to 4 ng/ml of TRAIL/Apo2L (Fig. 1A,B). Caspase activity was measured by cleavage of zVAD-PNA, a substrate of pan-caspase. As shown in Fig. 5, the caspase activity in TRAIL/Apo2L-treated HeLa cells increased over 10-fold compared with untreated cells. In addition, the pretreatment with 10 μM of zVAD-fmk blocked caspase activation. These results suggest that TRAIL/Apo2L-induced apoptosis occurs through the activation of common executors of apoptosis such as caspase-3.

To investigate the intracellular mechanism of caspase activation in TRAIL/Apo2L-induced cytotoxicity, we examined the effect of pretreatment with GSH or SB203580 on

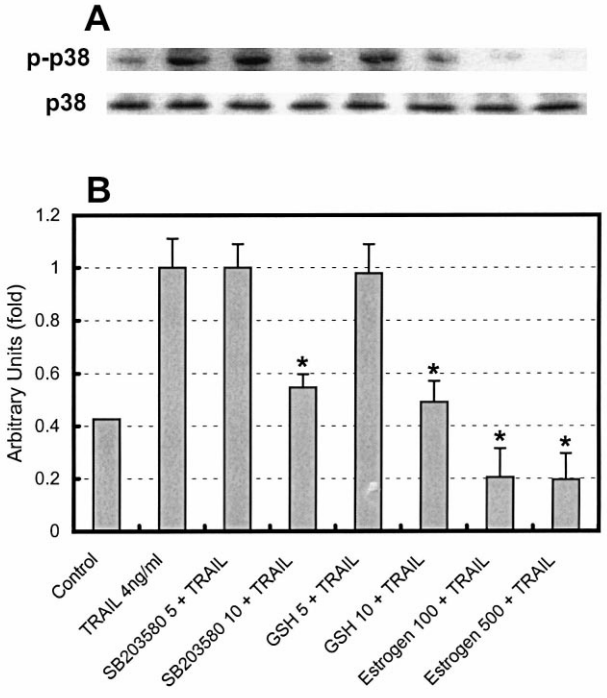

Fig. 4. Effect of SB203580, GSH or estrogen on TRAIL/Apo2L-activated p38 MAP kinase. A: HeLa cells were untreated (control; lane 1). Cells were left untreated (lane 2) or incubated with either 5 μM of SB203580 (lane 3), 10 μM of SB203580 (lane 4), 5 μM of GSH (lane 5), 10 μM of GSH (lane 6), 100 nM of estrogen (lane 7) or 500 nM of estrogen (lane 8) for 2 h, followed by treatment with 4 ng/ml of TRAIL/Apo2L for 2 h. The phosphorylation state of p38 MAP kinase was detected by Western blotting with a phosphospecific p38 MAP kinase antibody (p-p38). The same samples were probed with p38 MAP kinase antibody as a loading control (p38). B: Quantification of A results. Total levels of p38 MAP kinase were used as an internal standard in quantification of phosphorylated p38 MAP kinase. The levels of p38 MAP kinase are expressed as arbitrary units of relative value. *The difference from the cells incubated with vehicle alone was statistically significant ($P < 0.05$).

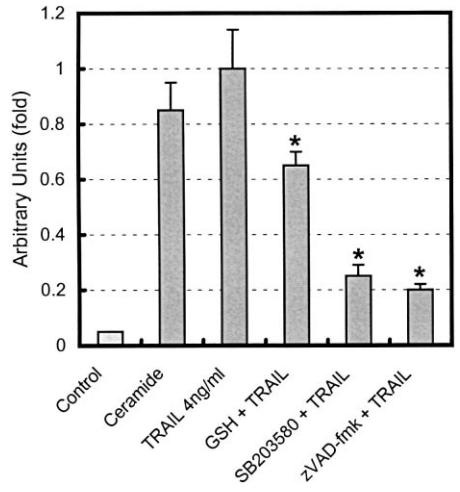

Fig. 5. Relative caspase activities induced by TRAIL/Apo2L after pretreatment with GSH, SB203580 or zVAD-fmk. Control was untreated HeLa cells (Control). Cells were left untreated (TRAIL) or incubated with either 10 μM of GSH (GSH+TRAIL), 10 μM of SB203580 (SB203580+TRAIL) or 10 μM of zVAD-fmk (zVAD-fmk+TRAIL) for 2 h, followed by treatment with 4 ng/ml of TRAIL/Apo2L for 6 h. 20 μg of cellular extracts were incubated with 0.25 mM zVAD-PNA, a substrate of pan-caspase, in 100 μl of total volume at 37°C for 1 h. Absorbance at 405 nm was measured with an ELISA Reader. Ceramide is positive control. Enzymatic activity is expressed as arbitrary units of relative value. Values are the mean ± S.E.M. of three separate experiments. *The difference from the cells treated with TRAIL/Apo2L alone was statistically significant ($P < 0.05$).

TRAIL/Apo2L-induced caspase activation. As shown in Fig. 5, the caspase activity was effectively reduced by pretreatment with 10 μM of GSH or SB203580. In contrast, the pretreatment with 10 μM of zVAD-fmk did not significantly reduce the accumulation of ROS and the activation of p38 MAP kinase induced by TRAIL/Apo2L (data not shown). These results indicate that ROS-activated p38 MAP kinase plays a role as an upstream regulator of caspase activation in TRAIL/Apo2L-induced apoptosis.

4. Discussion

Because TRAIL/Apo2L selectively induces apoptosis in tumor but not in normal cells, it has shown great potential to be a valuable tumor therapeutic agent [28]. However, the intracellular mechanisms of TRAIL/Apo2L-induced apoptosis are not fully understood. Thus, we tried to investigate the intracellular signaling of TRAIL/Apo2L. Our results show that ROS is involved in TRAIL/Apo2L-induced apoptosis in HeLa cells. TRAIL/Apo2L-induced apoptosis is inhibited by antioxidants such as GSH or estrogen, and this inhibition is correlated with a decrease in TRAIL/Apo2L-induced ROS formation. TRAIL/Apo2L-induced ROS generation and/or electrostatic incongruities may obstruct mitochondrial metabolism, altering the balance of biochemical chains of events, leading to a further increase in ROS generation, consequently entering into an intractable cycle of toxic events, eventually leading to cellular apoptosis.

In this study, we demonstrate that treatment of HeLa cells with TRAIL/Apo2L results in a rapid phosphorylation of p38 MAP kinase. Activation of p38 MAP kinase occurs within 0.5 h after exposure to TRAIL/Apo2L, which is prior to the onset of apoptosis. Also, p38 MAP kinase activity was functionally inhibited by SB203580. This inhibitor was reported to be highly specific for p38 MAP kinase both in vitro and in vivo [29]. A number of studies indicate that activation of p38 MAP kinase plays a crucial role in apoptosis induced by various stimuli. For example, activation of p38 MAP kinase is required for apoptosis induced by nerve growth factor depletion in PC12 neuronal cells [30]. In U937 and Jurkat cells, interfering with p38 MAP kinase has been shown to block apoptosis induced by ceramide and UV radiation [22,23]. Activation of p38 MAP kinase has also been implicated in anticancer drug-induced apoptosis [31]. Thus, the pretreatment of SB203580 was effective in preventing TRAIL/Apo2L-induced apoptosis, suggesting that p38 MAP kinase is required for the apoptosis induced by TRAIL/Apo2L.

Our results also show that the activation of p38 MAP kinase induced by TRAIL/Apo2L is effectively inhibited by pretreatment of GSH and estrogen, implicating that TRAIL/Apo2L-induced ROS acts upstream of p38 MAP kinase and participate in activation of p38 MAP kinase. Previous studies have shown that p38 MAP kinase is responsive to ROS and involved in apoptosis [14]. For example, nitric oxide induces the activation of p38 MAP kinase in HL-60 cells [32] and H_2O_2 preferentially stimulates the phosphorylation of p38 MAP kinase in vascular cells [33]. Recently, another study has reported that p38 MAP kinase is activated by ROS-activated STE20-related protein kinases [34].

Previous studies have shown that the caspase inhibitor effectively prevents TRAIL/Apo2L receptor- [35] and Fas-mediated apoptosis [36], suggesting that caspase plays an essential role in TRAIL/Apo2L receptor- and Fas-mediated signaling. In this study, we show that the pretreatment with zVAD-fmk could completely inhibit TRAIL/Apo2L-induced apoptosis in HeLa cells. However, this inhibition of caspase activities did not attenuate ROS generation and p38 MAP kinase activation by TRAIL/Apo2L, suggesting that caspases act downstream of ROS and p38 MAP kinase. Also, the treatment of HeLa cells with antioxidants or SB203580 attenuates the activation of caspases induced by TRAIL/Apo2L. Therefore, our results indicate that caspases which are activated by phosphorylated p38 MAP kinase may play an essential role in TRAIL/Apo2L-induced apoptosis.

In summary, this report is the first to show that TRAIL/Apo2L-induced apoptosis is mediated by ROS-activated p38 MAP kinase followed by caspase activation in HeLa cells. Understanding this intracellular mechanism of TRAIL/Apo2L-induced apoptosis may help to optimize TRAIL/Apo2L as a therapeutic agent in cancer.

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