Abstract. In cervical cancer, p53-induced apoptosis is abrogated by human papilloma virus (HPV)-derived oncoprotein E6. Although tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) provides tumor-specific apoptosis in various cancers, including cervical cancer, the sensitivity differs depending on the cell lines. Signal transducer and activator of transcription 3 (STAT3) is a hub molecule that shifts the cellular fate to apoptosis or survival in response to cellular stresses. However, the contribution of STAT3 activity to TRAIL-induced apoptosis in cervical cancer remains unknown. We examined the TRAIL sensitivity in cervical cancer cells, using TRAIL-resistant (SiHa) and -sensitive (CaSki) cervical cancer cell lines and focused on STAT3 function involving the apoptotic pathway. STAT3 was inactivated by TRAIL stimulation in the CaSki cell line, but not in the SiHa cell line. We then inhibited STAT3 expression in the SiHa cell line using siRNA and suppressed STAT3 activity using a STAT3 inhibitor; both these treatments sensitized TRAIL-induced apoptosis in the SiHa cell line. Furthermore, the SiHa cells were exposed to tunicamycin (TM), an endoplasmic reticulum (ER) stress inducer that inactivates STAT3, with or without TRAIL. Accompanied by STAT3 inactivation, TM pretreatment significantly enhanced TRAIL-induced apoptosis. We therefore concluded that TRAIL-induced apoptosis was regulated by STAT3 in response to TRAIL stimulation. Our results also suggest that STAT3 inhibition increases the sensitivity of malignancies, particularly HPV-related cancer, to TRAIL-based therapy.

Introduction

Cervical cancer is the most common carcinoma caused by oncogenic human papillomavirus (HPV). Globally, it accounted for an estimated 528,000 new cancer cases worldwide and 266,000 deaths in 2012 (1) even though HPV vaccination programs have been implemented worldwide. Conventional therapy for advanced cervical cancer is radiotherapy, chemotherapy, or both. However, some populations with chemo-resistance have poor prognoses. Alternative therapeutics including molecule-targeting agents have not yet been developed for cervical cancer.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily and has received attention for its role as an antitumor agent that has few cytotoxic effects on normal cells (2-4). Two major pathways exist downstream of TRAIL signaling: pro-apoptosis and pro-survival signaling (5). The apoptosis pathway directly induces the TRAIL-R1/2-DISC (death-inducing signaling complex)-induced activation of caspase-8 or triggers apoptosis indirectly via a mitochondrial pathway. On the other hand, the pro-survival pathway induces the activation of NF-κB or JNK/p38 signaling and increases the expression of anti-apoptotic or proliferation stimulatory proteins (5). The fate of TRAIL signaling is controlled by the balance between pro-apoptotic and pro-survival signaling, which varies among tumor cell types.

In cervical cancer cells, p53-induced apoptosis is abrogated through the degradation of p53 by the HPV E6 oncoprotein (6) and thus, the other types of apoptosis pathway such as
TRAIL-induced apoptosis pathway appear to play a major role in cell programmed death. Overcoming TRAIL resistance has a potential to improve outcome in treating cervical cancer. A previous study reported that sensitivity to TRAIL-induced apoptosis differed among cell lines and that the proteasome inhibitor MG132 sensitized TRAIL-induced apoptosis in cervical cancer cells by upregulating death receptor (DR) 4/5 and inactivating X-linked inhibitor of apoptosis (XIAP) (7). However, the biological mechanisms preventing TRAIL-induced apoptosis in several cell lines have not yet been elucidated in detail.

Signal transducer and activator of transcription 3 (STAT3) is activated by tyrosine phosphorylation in response to various cytokine stimuli (8-10). STAT3 is induced by various types of cellular stresses such as hypoxia, reperfusion, and ultraviolet (UV) (11), which often occur in the tumor microenvironment (TME). STAT3 and its downstream p38 mitogen-activated protein kinase (MAPK) signaling pathway are well-known regulators of apoptosis or survival of damaged cells (11-13). The constitutive activation of STAT3 has been reported in various types of malignancies, including cervical cancer (14-18). Notably, the inhibition of STAT3 has been shown to shift some apoptosis-resistant cells to TRAIL-induced apoptosis (19,20). Numerous reports have demonstrated that several components with the potency to suppress STAT3 activation such as histone deacetylase (HDAC) inhibitors (21-25), resveratrol (26-30), and curcumin (31-35) enhanced TRAIL-induced apoptosis in TRAIL-resistant cells.

Due to the importance of the STAT3 pathway inhibition and TRAIL-induced apoptosis especially in cervical cancer therapy, we attempted to elucidate the mechanisms responsible for TRAIL-induced apoptosis with a focus on STAT3 activity.

Materials and methods

Antibodies and reagents. The following antibodies were used at the dilutions indicated. In western blotting, mouse anti-human α-Tubulin sc-8035 (1:500) purchased from Santa Cruz Biotechnology (TX, USA), mouse anti-human total STAT3 (124H6) CS#9139 (1:1,000), rabbit anti-human phospho-STAT3 (Tyr705) (D3A7) CS#9145 (1:1,000), purchased from Cell Signaling Technologies (MA, USA). The STAT3 inhibitor S31-201 was purchased from Santa Cruz Biotechnology. Recombinant human TRAIL was purchased from R&D Systems (Minnetonka, MN, USA).

Cell cultures. The cervical cancer cell lines SiHa and CaSk were maintained in Dulbecco's modified Eagle's medium (Wako, Osaka Japan) with 10% fetal bovine serum (FBS, Life Technologies, CA, USA) and antibiotics (antibiotic-antimycotic mixed stock solution, Nacalai Tesque, Kyoto, Japan). Cells were grown in a humidified tissue culture incubator at 37˚C in 5% CO2.

Cell proliferation assay. Cell proliferation assays were performed to analyze the effects of TRAIL, the STAT3 inhibitor S31-201, tunicamycin on cell proliferation. Five thousand cells were seeded on 96-well plates. Cell Counting Kit-8 (CCK-8) with the tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (Dojindo, Osaka, Japan) was used and quantified by monitoring changes in absorbance at 450 nm, which were normalized relative to the absorbance of control cells. The lengths of the treatments are described with each result.

Detection of apoptosis by staining with Annexin V-FITC. Cells (4x105) were cultured in 6-well plates for 24 h before being treated. SiHa was also pretreated with S31-201 or tunicamycin for 24 h with an additional 15-18 h of the TRAIL treatment. Cells were trypsinized, washed with PBS, and then analyzed after double staining with an Annexin V A apoptosis Detection kit (Abcam, MA, USA). The apoptotic cell population was assessed using flow cytometry.

Immunoblotting. Total cellular extracts were prepared by lysing cells from dishes in lysis buffer (#9803, Cell Signaling Technologies) containing protease inhibitor cocktail (Nacalai Tesque) and phosphatase inhibitor cocktail (Roche, Mannheim, Germany) on ice for 5 min, and then sonicated briefly. Cells were centrifuged at 14,000 rpm at 4˚C for 10 min. The supernatant was used in subsequent analyses. In SDS-PAGE, 20 µg of the protein lysate with 6X sample buffer (Nacalai Tesque) was loaded into each well. In immunoblotting, 0.45-µm PVDF membranes (Merck Millipore, Darmstadt, Germany) were used. The membranes were blocked in 5% milk/TBS-T (TBS containing 0.1% Tween-20) at room temperature for 1 h followed by an incubation with the primary antibodies diluted in 5% milk/TBS-T or 5% BSA/TBS-T for the appropriate time period indicated in the manufacturer's instructions. After several washes with TBS-T, the membranes were incubated with secondary antibodies conjugated with HRP in 5% milk/TBS-T at room temperature for 1 h. Blots were developed using Immobilon Western Chemiluminescent HRP substrate (Merck Millipore) according to the manufacturer's instructions.

Transfections. Small interfering RNA transfections were performed using Stealth RNAi against STAT3 (HSS186130, HSS186131, and HSS110279) and non-targeting siRNA (Stealth RNAi™ siRNA Negative Control, Med GC, Life Technologies) as a control. When cells were 60-70% confluent, transfections were performed using Lipofectamine RNAiMAX (Life Technologies), Opti-MEM reduced serum medium (Life Technologies), and a final concentration of 20 nmol/l of siRNAs, according to the manufacturer's instructions. After 5 h of incubation, the transfection medium was changed to normal culture medium without antibiotics. Cells were incubated for 48 h and then analyzed for each experiment. The transfection sequence was repeated at least 3 times.

RT-quantitative PCR. Total RNA was extracted from cells using Blood/Cultured Cell Total RNA Mini kit (Favorgen, Ping Tung, Taiwan), followed by reverse transcription. cDNA was amplified for 40 cycles in a Light Cycler 480 (Roche). Expression of CHOP was normalized by GAPDH mRNA as an internal standard, and spliced XBP1 (sXBP1) was normalized by total XBP1 (tXBP1) calculated by the ΔΔCq method. The primer pairs were as follows: human total XBP1 (tXBP1) 5'-GGCATCCTTT
STAT3 has been reported to be involved in apoptosis resistance in various cancers (36-38). We investigated the involvement of STAT3 in TRAIL sensitivity between CaSki and SiHa (Fig. 1). Western blotting revealed that the expression levels of total STAT3 (tSTAT3) were similar in both cell types regardless of the TRAIL stimulation. The phosphorylation of STAT3 (pSTAT3) was suppressed in CaSki by TRAIL, but tend to be upregulated or remained at the same level in SiHa (Fig. 1, upper panels). Six independent experiments revealed that the protein level of pSTAT3 decreased significantly (P=0.0003) by TRAIL in CaSki, but not in SiHa (P=0.0957) (Fig. 1, lower panels). These results indicated that the modulation of STAT3 activity by TRAIL might be involved in the different responses to the TRAIL stimulation.

siSTAT3 enhances TRAIL-induced apoptosis. In order to examine the role of STAT3 in resistance to TRAIL-induced apoptosis, optimized siRNA for STAT3 (siSTAT3) or control siRNA was transduced into SiHa (Fig. 2). Total STAT3 (tSTAT3) as well as phosphorylation of STAT3 (pSTAT3) expression were sufficiently knocked-down at the protein level by siSTAT3 in SiHa (Fig. 2A). STAT3 knockdown (siSTAT3) and control cells were exposed to TRAIL and then assessed for apoptosis. Although the TRAIL stimulation induced apoptosis in control and siSTAT3 cells, the extent of apoptosis observed was markedly higher in siSTAT3 cells [21.2(±2.6)%] than in control cells [8.7(±0.44)%] (Fig. 2B). The increase induced in apoptosis by the knockdown of STAT3 did not occur in cells not exposed to TRAIL. These results indicated that the knockdown of STAT3 enhanced sensitivity to TRAIL-induced apoptosis in SiHa.

Next, we investigated the effect of STAT3 suppression on cisplatin (CDDP)-induced apoptosis in the SiHa cells, as CDDP is one of the standard chemotherapeutics for advanced or recurrent cervical cancer (39). Unlike TRAIL-induced apoptosis, STAT3 inhibition did not enhance CDDP-induced apoptosis in the SiHa cells (data not shown).

The STAT3 inhibitor enhances sensitivity to TRAIL-induced apoptosis. As a molecular-targeting agent in a clinical setting, S3I-201 is an attractive STAT3 inhibitor which inhibits STAT3 dimerization, DNA binding and transcriptional activities (40). We hypothesized that S3I-201 may enhance sensitivity to TRAIL-induced apoptosis in cells resistant to TRAIL. Apoptosis was examined in TRAIL-stimulated SiHa cells treated with or without the STAT3 inhibitor S3I-201 (Fig. 3A). Increases of less than 5% were observed in the proportion of apoptotic cells by the TRAIL stimulation without exposure to S3I-201. TRAIL-stimulated SiHa showed a five-fold increase [4.3(±0.97)% to 23(±2.1)%] in the proportion of apoptotic cells following exposure to 100 µM of S3I-201 (Fig. 3A).
Furthermore, SiHa cells were exposed to S3I-201 at different doses (25, 50 and 100 µM) with or without a sequential 100 ng/ml of TRAIL stimulation, and cell viability at each dose was blotted on the curve (Fig. 3B). Although S3I-201 alone suppressed cell viability at doses >50 µM, the combination of S3I-201 and TRAIL more effectively suppressed cell viability in SiHa. These results indicated that the STAT3 inhibitor, synergistic with TRAIL, enhanced sensitivity to apoptosis in TRAIL-resistant SiHa cell line.

**Tunicamycin sensitized TRAIL-induced apoptosis by suppressing STAT3 activation in SiHa.** Previous studies demonstrated that several ER stress inducers suppressed STAT3 phosphorylation (41,42). We hypothesized that ER stress inducers may sensitize cells to TRAIL-induced apoptosis through the inactivation of STAT3. The TRAIL-resistant cell line, SiHa, was exposed to different doses of the ER stress inducer, tunicamycin (TM) (0.01, 0.05, 0.1, 0.5 and 1.0 µM) to examine the phosphorylation of STAT3 and TRAIL-induced apoptosis. We confirmed that TM upregulated C/EBP homologous protein (CHOP) and X-box-binding protein-1 (XBP1) splicing levels in a dose-dependent manner, indicating that TM activated an unfolded protein reaction (UPR): ER stress branches (Fig. 4A). Western blotting for pSTAT3 and tSTAT3 demonstrated that TM successfully suppressed the activation (phosphorylation) of STAT3 in a dose-dependent manner (Fig. 4B).

SiHa was then exposed to TM at different doses (0.01, 0.05, 0.1, 0.5 and 1.0 µM) for 24 h with or without a sequential 100 ng/ml of TRAIL stimulation, and cell viability was assessed using CCK-8 (Fig. 5A). TM reduced cell viability to less than 50% at doses greater than 0.1 µM in the absence of TRAIL (Fig. 5A). Since TM increased spliced XBP1 and
CHOP, which play central roles in ER stress-mediated apoptosis (43) (Fig. 4A), decreases in cell viability appeared to reflect TM-induced ER stress-mediated apoptosis. However, cell viability curves revealed that TM in combination with TRAIL had more suppressive effect on the cell viability compared with TM alone. In the presence of TRAIL, viable cells were barely detected at the same doses of TM (Fig. 5A). We also examined the proportion of apoptotic cells among SiHa exposed to TRAIL and/or TM at different doses (Fig. 5B). The proportion of apoptotic cells was only 5.9(±0.22)% in SiHa exposed to TRAIL alone, but increased to 47.1(±3.4)% and 81.0(±0.2)% in SiHa exposed to TRAIL in combination with TM at 0.05 and 0.1 µM, respectively. Exposure to TM alone induced apoptosis in 5.8(±0.68)% and 12.4(±0.48)% of cells at these doses. Therefore, the combination of TM and TRAIL had a synergistic effect on the induction of apoptosis.

Discussion

In this study, we showed that STAT3 activation was suppressed by TRAIL in the TRAIL-sensitive cell line CaSki, but not in the TRAIL-resistant SiHa cell line. The inhibition of STAT3 expression using siRNA technology and the suppression of STAT3 activity using a STAT3 inhibitor increased the sensitivity of the SiHa cells to TRAIL-induced apoptosis. Furthermore, an ER stress inducer (TM) also effectively increased their sensitivity to TRAIL-induced apoptosis accompanied by STAT3 inactivation. These results indicated that STAT3 regulated the TRAIL sensitivity in the SiHa cells.

We first examined the differences in the basal expression of apoptosis-related molecules including apoptosis-related receptors. However, no marked differences were observed in the expression levels of apoptosis-related molecules or TRAIL receptors (data not shown). In contrast, western blotting revealed a difference in the phosphorylation of STAT3 with TRAIL stimulation (Fig. 1), whereas other major pro-apoptotic signaling pathways remained unchanged (data not shown). These results indicate that STAT3 activity might be involved in the difference in the responses of the two cell lines to TRAIL stimulation, which is consistent with previous findings showing that the inhibition of STAT3 signals sensitizes TRAIL-induced apoptosis (41,42). Previous studies reported that several components that have the potency to suppress STAT3 shift the fate of some apoptosis-resistant malignant cells to TRAIL-induced apoptosis. In these studies, a JAK2 inhibitor, histone deacetylase (HDAC) inhibitor, resveratrol, and curcumin were used as inducers of TRAIL-induced apoptosis (21-35). Taken together, these data suggested that STAT3 might be the central molecule in the resistance of the SiHa cells to TRAIL. In this study, we further confirmed that both suppression of STAT3 expression using siRNA technology and inhibition of STAT3 activation using the STAT3
inhibitor S3I-201 increased the TRAIL-induced apoptosis in the SiHa cells (Figs. 2 and 3). Oncogenes and p53 are the central regulators of the apoptosis induction in cervical cancer. A previous report demonstrated that knockdown of STAT3 suppressed the expression of viral E6 and E7 oncoproteins as well as upregulated p53 expression (44). Upregulation of p53 might also enhance the apoptosis-inducing function of the STAT3 knockdown in our model. These results indicated that targeting STAT3 expression or activity combined with TRAIL stimulation could be a strategy for cervical cancer treatment.

ER stress-mediated apoptosis occurs following the disruption of the UPR balance under prolonged ER stress. UPR branches (PERK, IRE1a, and ATF6) are well known to cross-talk with each other and various cellular signaling pathways (45). However, it currently remains unclear whether STAT3 preferentially involves UPR or ER stress-mediated apoptosis. Several studies have shown that some ER stress inducers suppress the phosphorylation of STAT3 (41,42). The results of this study showed that 0.1 μM of tunicamycin (TM) upregulated CHOP, which plays a central role in ER stress-mediated apoptosis (43), although it induced apoptosis in only 10% of the treated cells (Fig. 5B). This result indicated that TM activated UPR, but did not induce ER stress-mediated apoptosis at this dose. In contrast, the combination of TRAIL and 0.1 μM of TM induced apoptosis in 80% of the treated cells (Fig. 5B), which suggested that the combination of TRAIL and TM had a synergistic effect on the induction of apoptosis. Although TM is known to itself upregulate DR4 or DR5 and sensitize cells to TRAIL-induced apoptosis (46-49), only a slight increase in DR5 mRNA level in the SiHa cells was observed after TM treatment in our experiments (data not shown). The inactivation of STAT3 by TM may be one of the key mechanisms to increase the sensitivity of SiHa cells to TRAIL-induced apoptosis, which possibly resulted in the synergistic effect observed.

In addition to the TRAIL-induced apoptosis pathway, we also investigated the effect of STAT3 suppression on the CDDP-induced apoptosis pathway. Although previous reports demonstrated that the inhibition of STAT3 enhanced the CDDP-induced apoptosis in various cancers (50,51), in our model, the inhibition of STAT3 did not enhance the CDDP-induced apoptosis. In the CDDP-induced apoptosis, DNA damage is induced by intra-strand or inter-strand cross-link between two adjacent G residues (52). This DNA damage induces p53-dependent apoptosis. However, HPV-positive cervical cancer lacks p53 expression and the DNA damage-induced apoptosis pathway is considered to be different from other types of cancer. The result suggested that STAT3 inhibition could be an effective therapeutic modality for cervical cancer when combined with TRAIL rather than with CDDP.

In conclusion, in this study, we showed that different TRAIL sensitivity among cell lines might be regulated by STAT3 activity and that the inactivation of STAT3 enhances the sensitivity of the cells to TRAIL-induced apoptosis, even in a TRAIL-resistant cancer cell line. Our results suggest the potential of STAT3 inhibition in combination with TRAIL-based therapy for cervical cancer.

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References


