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Distinct Roles for JNK1 and JNK3 During TNF-α- or Etoposide-Induced Apoptosis in HeLa Cells

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Here, we show that JNK1 and JNK3 have different roles in TNF-α- or etoposide-induced apoptosis in HeLa cells. Dominant negative JNK1 inhibited TNF-α- or etoposide-induced apoptosis, while dominant negative JNK3 promoted TNF-α- or etoposide-induced apoptosis. During TNF-α-induced apoptosis, JNK1 was activated in a biphasic manner, exhibiting both transient and sustained activity, whereas JNK3 was activated early and in a transient manner. The role of JNK3 activation was an anti-apoptotic effect, while the role of JNK1 activation was a pro-apoptotic effect. These results suggest that the anti-apoptotic mechanism of JNK3 in TNF-α-induced apoptosis originates before the apoptotic machinery is triggered.

INTRODUCTION

Apoptosis is regulated by numerous intracellular signaling pathways, including the c-Jun N-terminal kinase (JNK) pathway (Davis, 2000), which is stimulated primarily by environmental stress and inflammatory cytokines (Weston and Davis, 2007). Tumor necrosis factor (TNF) induces apoptosis through the activation of JNK-mediated signaling pathways. The binding of TNF to its cognate receptor, tumor necrosis factor alpha receptor 1 (TNFR1), results in the recruitment of the adapter protein, TNFR1-associated death domain protein (TRADD) to the cytoplasmic domain of the receptor. TRADD then recruits Fas-associated death domain protein (FADD), TNFR-associated factor 2 (TRAF2), and receptor-interacting protein 1 (RIP1) to the receptor complex (Chen and Goeddel, 2002). FADD-mediated activation of caspase-8 leads to apoptosis (Varfolomeev et al., 1998; Yeh et al., 1998), while TRAF2- and RIP1-mediated activation of the NF-κB pathway suppresses apoptosis in response to TNF (Kelliher et al., 1998; Yeh et al., 1997). Thus, the cellular response to TNF reflects in part, a balance between these two opposing pathways (Varfolomeev and Ashkenazi, 2004).

There are three isoforms of JNK (JNK1, 2 and 3) that arise as a result of alternative splicing (Davis, 2000). JNK1 and JNK2 are present in most tissues, whereas JNK3 is selectively expressed in the nervous system and heart. JNK3 has been preferentially implicated in stress-induced neuronal apoptosis Kuan et al. (2003). However, overall, the precise roles of the different isoforms of JNK are controversial. JNKs can function as pro-apoptotic or anti-apoptotic kinases (Minden et al., 1994). For example, the restoration of transient JNK activity in MCF-7 cells blocks TNF-α-induced apoptosis, suggesting that the activation of JNK during TNF-α-induced apoptosis is critical for cell survival (Tang et al., 2002). Recently, it was reported that the phosphorylation of JNK3 by CDK5 reduces c-Jun phosphorylation and prevents neuronal apoptosis (Li et al., 2002).

Here we have shown that a dominant negative form of JNK1, DN-JNK1, inhibits TNF-α- or etoposide-induced apoptosis in HeLa cells, while a dominant negative form of JNK3, DN-JNK3, promotes apoptosis. TNF-α-induced JNK3 activation was an early event following exposure to TNF-α, and was functionally distinct from the activation of JNK1.

MATERIALS AND METHODS

Reagents

TNF-α was purchased from Biosource International and stored at -20°C. Cycloheximide was obtained from Sigma-Aldrich (USA), dissolved in phosphate buffered saline (PBS) at a concentration of 3 mg/ml and then stored at -20°C. Protein G-agarose beads were purchased from Upstate Biotechnology (USA) and the caspase-3 substrate, Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC), was purchased from BD PharMingen (USA).

Cell culture and transfection

Wild type HeLa cells and stable HeLa cell transfectants expressing JNK binding domain (JBD) of JNK-interacting protein-1, JNK3, or neomycin (neo) as a control were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics/antimycotics (Gibco-BRL, USA) at 37°C in a 5% CO_2 atmosphere. Transient transfections were performed in 6-well plates using Polyfect (Qiagen, USA). Unless otherwise noted, cells were seeded at a density of 2×10^5 cells per well and transfected using a total of 4 μg of DNA. Transfections were allowed to proceed for

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24-40 h, and then cells were treated with TNF-α (3 ng/ml) and cycloheximide (3 μg/ml). Cell viability, caspase-3 activity and Western blot analysis were then carried out.

To construct stable cell lines, HeLa cells were transfected with pCDNA3-Flag-JNK3 or pCDNA3-Flag-JBD. Transfected cells were grown in culture media containing 800 μg/ml G418 for 2 weeks. Surviving clones expressing JNK3 or JBD were designated as either JNK3-HeLa or JBD-HeLa cells, respectively. Stable cell clones were maintained in DMEM supplemented with 10% FBS and 400 μg/ml G418.

Western blot analysis

Western blot analysis was performed according to standard methodology. Cells were lysed in lysis buffer (0.5% Triton X-100, 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 50 mM β-glycerophosphate, 25 mM NaF, 1 mM Na_3VO_4, 100 μg/ml PMSF, protease inhibitor cocktail) for 1 h. Lysates were subjected to SDS-PAGE and then proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). The membrane was incubated in a solution of 5% non-fat dried milk (Carnation) in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4-$7H_2O$, 1.4 mM KH_2PO_4, pH 7.4) containing 0.05% Tween 20 (PBS-T), and then incubated with one of the following primary antibodies (1:1000 dilution), as indicated: mouse monoclonal anti-phospho c-Jun, mouse monoclonal anti-phospho JNK, mouse monoclonal anti-phospho JNK1, rabbit polyclonal anti-JNK1, rabbit polyclonal anti-PARP, rabbit polyclonal anti-actin, mouse monoclonal anti-tubulin (Santa Cruz Biotechnology, USA), mouse monoclonal anti-Smac/DIABLO, rabbit polyclonal anti-phospho SAPK (Cell Signaling Technology, USA), rabbit polyclonal anti-JNK3 (Upstate Biotechnology Inc, USA), goat polyclonal anti-JNK3 (Santa Cruz Biotechnology, USA) or mouse monoclonal anti-Flag (Sigma-Aldrich). Immunoreactive proteins were visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies and the ECL system (iNtRON Biotechnology, Korea).

Subcellular fractionation

HeLa cells were washed with PBS and then resuspended in 3 volumes of ice-cold buffer A (20 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail) containing 250 mM sucrose. The lysates were subjected to centrifugation at 1,000 × *g* for 5 min at 4°C. The resulting pellet contained nuclei, cellular debris and intact cells, and the supernatant contained the cytosolic fraction, including mitochondria. The supernatant was collected and subjected to centrifugation at 10,000 × *g* for 20 min at 4°C to remove the mito chondria. The supernatant, including the cytosolic fraction, was subjected to a second round of centrifugation at 100,000 × *g* for 1 h to remove residual mitochondria.

Immunoprecipitation

JNK3 stable transfectants were lysed in lysis buffer and then total cell lysate (1,500 μg) was subjected to immunoprecipitation overnight at 4°C using an anti-FLAG antibody (1 μg). Antigen-antibody complexes were captured by incubation with protein G agarose beads (100 μl) for 3-4 h at 4°C. The beads were washed three times with lysis buffer, and then subjected to Western blot analysis.

Caspase-3 activity assay

Caspase-3 activity was assayed using Ac-DEVD-AMC as a substrate, according to the manufacturer's instructions, with some modifications. Briefly, cell lysate (10 μg) was added to reaction buffer (20 mM HEPES, pH 7.5, 10% glycerol, 2 mM DTT) containing 25 μM Ac-DEVD-AMC in a 96-well plate, and then incubated at 37°C for 1 h. The fluorescence of the caspase-3 cleavage product was measured using a SpectraFluor F129003 system (TECAN US, USA) at an excitation wavelength of 360 nm and an emission wavelength of 465 nm.

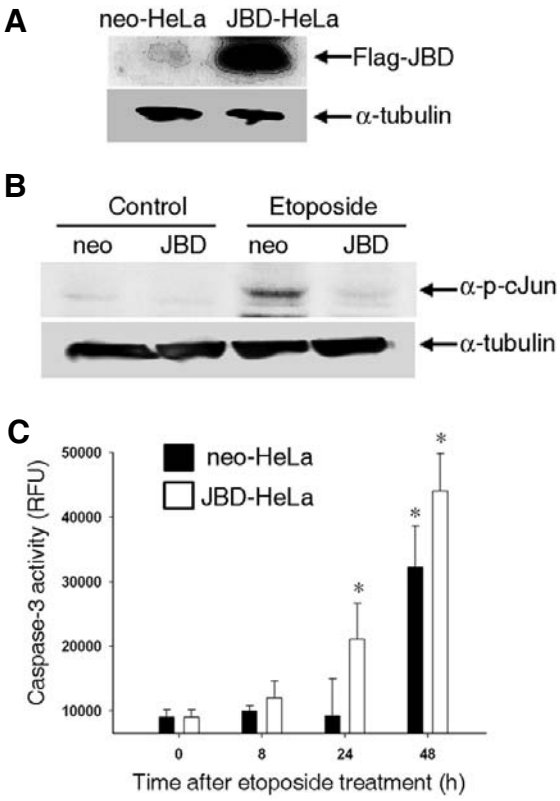

Fig. 1. Apoptosis is increased in response to etoposide in HeLa-JBD stable transfectants as compared to control, neomycin transfectants. (A, B) The protein levels and inhibitory activity of JBD were examined by Western blot using an anti-Flag antibody directed against phosphorylated c-Jun, respectively. Loading control was examined with anti-tubulin antibody. (C) JBD stable transfectants and control neo stable transfectants were treated with etoposide for increasing amounts of time, and the activity of caspase-3 was measured. *$P < 0.05$ versus control.

RESULTS

To determine the effect of JNK activation on apoptosis, stable transfectants of HeLa cells that expressed JBD (JBD-HeLa), or control neo transfectants (neo-HeLa) (Fig. 1A) were treated with the apoptosis-inducing agent etoposide. JNK activity was abrogated in JBD stable transfectants (Fig. 1B), and caspase-3 activity was more pronounced in JBD transfectants than in control cells following exposure to etoposide (Fig. 1C). These results suggested that JNK is involved in survival signaling pathways in HeLa cells.

To determine which isoform(s) of JNK were involved in survival signaling, HeLa cells were transfected with expression vectors for DN-JNK1 or DN-JNK3 (Fig. 2A), and then caspase-3 activity and cell viability were measured. The expression level of DN-JNK1 or DN-JNK3 was measured by western blotting with anti-Flag (Fig. 2A, first panel), JNK1 (Fig. 2A, second

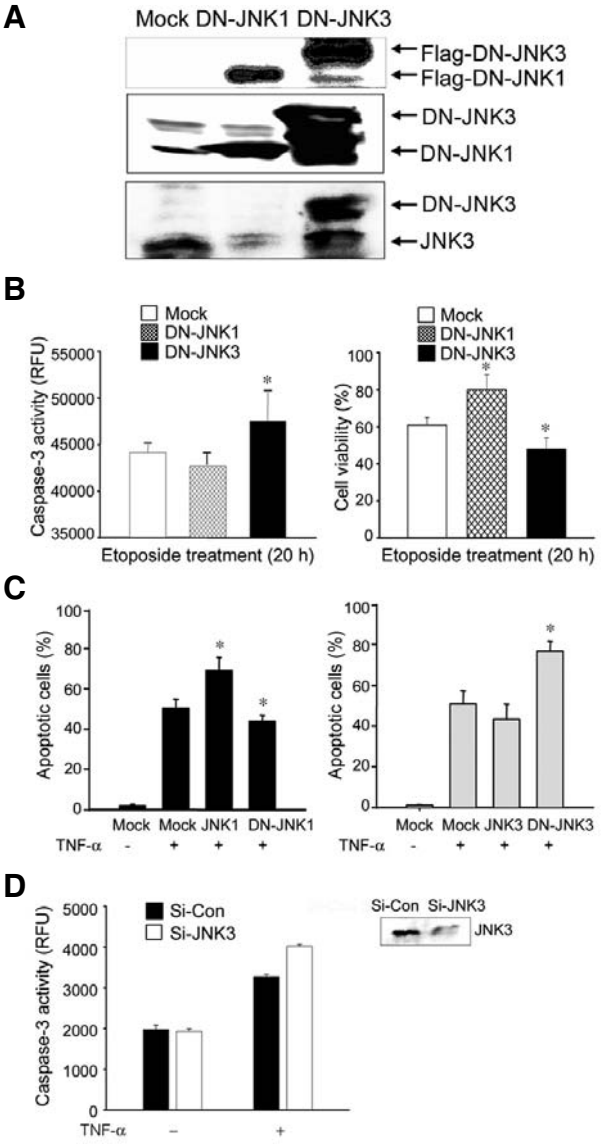

Fig. 2. JNK1 and JNK3 have different effects in etoposide-induced apoptosis. (A) Expression vectors for Flag-epitope-tagged DN-JNK1 (Flag-DN-JNK1) or Flag-DN-JNK3 were transiently transfected into HeLa cells, and the expression levels of DN-JNK1 and DN-JNK3 were examined by Western blot using an anti-Flag, anti-JNK1 or anti-JNK3 antibodies. (B) Cells were transfected with expression vectors for Flag-DN-JNK1 or Flag-DN-JNK3, or pCDNA3 as a control (Mock). Thirty-two h after transfection, cells were treated with etoposide for 20 h, and then caspase-3 activity and cell viability were measured. The data represents the averages of two independent experiments. *$P < 0.05$ versus Mock. (C) HeLa cells were co-transfected with pCS2+GFP and an expression vector for Flag-JNK1, Flag-JNK3, Flag-DN-JNK1, or Flag-DN-JNK3, or Mock-transfected as a control, using Polyfect. Thirty-two h after transfection, cells were treated with TNF-α and cycloheximide for over 6 h. The number of GFP-positive apoptotic cells with evidence of membrane blebbing was counted. The results represent the means and standard deviation of at least three independent experiments. *$P < 0.05$ versus Mock. (D) siRNA-JNK3 (Santa Cruz, 100 pmol/ml) was transfected by electroporation method (MicroPorator). TNF-α and cyclohexamide were treated for 10 h and the cells were harvested. Lysates were subjected to caspase-3 activity assay.

panel) or JNK3 (Fig. 2A, bottom panel). Since JNK1 antibody used in the experiments catches both JNK1 and JNK3, the both endogeneous level of JNK 1/3 and transfected level of DN-JNK1/DN-JNK3 were detected by JNK1 antibody (Fig. 2A, second panel). While, the endogeneous level of JNK3 and transfected level of DN-JNK3 were only detected by specific JNK3 antibody (Fig. 2A, bottom panel).

DN-JNK3 promoted caspase-3 activity and apoptotic cell death in response to etoposide and TNF-α (Figs. 2B and 2C, right), whereas DN-JNK1 attenuated etoposide- or TNF-α-induced apoptotic cell death (Figs. 2B and 2C, left). In the presence of etoposide or TNF-α, cell viability was higher in cells that overexpressed DN-JNK1 than in mock or JNK1-expressing cells, whereas cell death was higher in DN-JNK3-expressing cells than in mock or JNK3-expressing cells. These results indicated that JNK3 plays a role in cell survival in HeLa cells. These results were confirmed by knockdown of JNK3 in HeLa cells. The suppression of JNK3 using Si-RNA promoted caspase-3 activity induced by TNF-α compared to that of Si-control (Fig. 2D).

Next, we investigated the time-course of JNK1 and JNK3 activation during TNF-α-induced apoptosis. Western blot analysis using an antibody that recognized both JNK1 and JNK3 revealed that JNK is activated in a biphasic manner following treatment with TNF-α with increased activation at 1 and 6 h (Fig. 3A, third panel). When we examined the time-course of activation of individual isoforms of JNK, we found that JNK1 is activated in biphasic manner, whereas JNK1 protein levels remained constant following treatment with TNF-α (Fig. 3A, third and fourth panel). In contrast, the protein levels of JNK3 increased at 1 h and then decreased for up to 8 h following TNF-α treatment (Fig. 3A, fifth panel).

Due to the low level of JNK3 in HeLa cells, we performed additional experiments in JNK3 stable transfectants. The PARP cleavage in HeLa cells occurred at 6 h after treatment of TNF-α (Fig. 3A, first and second panel), whereas that of JNK3-HeLa cells slightly appeared at 8 h after treatment of TNF-α (Fig. 3A, eighth panel). JNK3 was activated at an early time point (1 h) during TNF-α-induced apoptosis (Fig. 3A, seventh panel) in JNK3 stable transfectants. The protein levels of JNK3 also increased 1 h after TNF-α exposure, and then decreased (Fig. 3A, eighth panel). These results were consistent with those obtained using wild-type HeLa cells (fifth panel).

We also investigated the time-course of caspase-3 activity in JNK3 stable transfectants, and found that the TNF-α-induced activation of caspase-3 was delayed in JNK3 transfectants as compared to control cells (Fig. 3B).

To determine the specific roles of JNK1 and JNK3 during apoptosis, we examined caspase-3 activity at early and late time points during TNF-α-induced apoptosis. Caspase-3 activity at the late time point (7 h) in JNK1 transfectants was elevated (Fig. 4A, right panel), while in DN-JNK1 transfectants, it was slightly inhibited as compared to mock transfectants (Fig. 4A, right panel). There was no difference in caspase-3 activity between JNK1 and DN-JNK1 transfectants at the early time point (5 h) (Fig. 4A, left panel). The release of Smac was also inhibited in DN-JNK1 transfectants at the late time point (7 h) (Fig. 4B). These results indicated that TNF-α-induced JNK1 activity is involved the later stages of apoptosis.

Caspase-3 activity at the early time point was inhibited in JNK3 transfectants, whereas it was elevated in DN-JNK3 transfectants as compared to mock transfectants (Fig. 4C, left panel). There was no difference in caspase-3 activity between JNK3 and DN-JNK3 transfectants at the late (7 h) time point (Fig. 4C, right panel). The release of Smac was also elevated in DN-

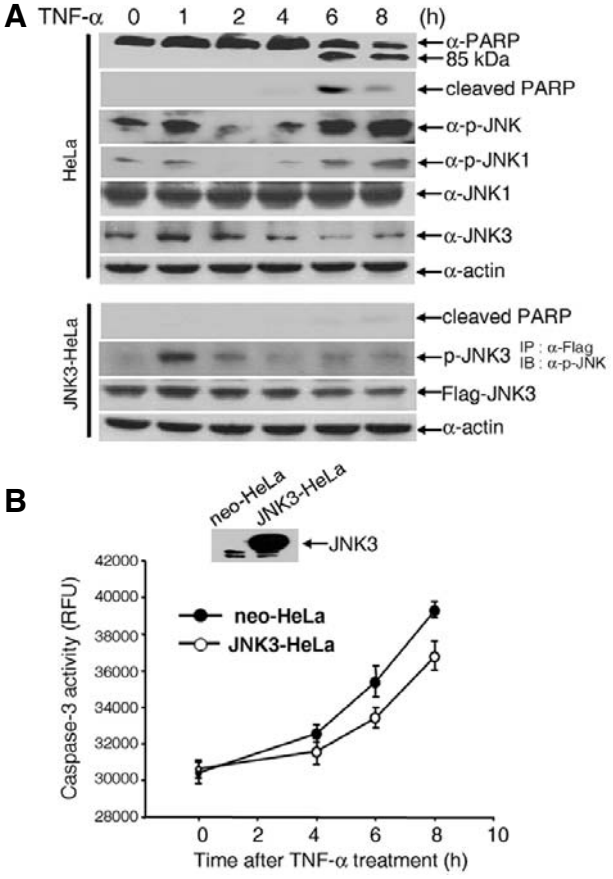

Fig. 3. JNK1 and JNK3 activities are up-regulated at different times during TNF-α-induced apoptosis. (A) Wild-type HeLa cells and JNK3 stable transfectants were harvested at the indicated periods of time after exposure to TNF-α and cycloheximide, and cellular extracts were analyzed by Western blot using anti-phospho JNK, anti-JNK1, anti-PARP, anti-actin and anti-JNK3 antibodies, as indicated. Cell lysates were prepared from JNK3 stable transfectants, and subjected to immunoprecipitation using an anti-Flag antibody. Immunoprecipitates were recovered with protein G-agarose beads and subjected to 15% SDS-PAGE, followed by Western blot using an anti-phospho SAPK antibody. (B) JNK3 or neo stable transfectants were treated with TNF-α and cycloheximide for the indicated times. Cells were harvested and caspase-3 activity was measured using Ac-DEVD-AMC as a fluorescent substrate. Data represent the means and standard deviation of two independent experiments.

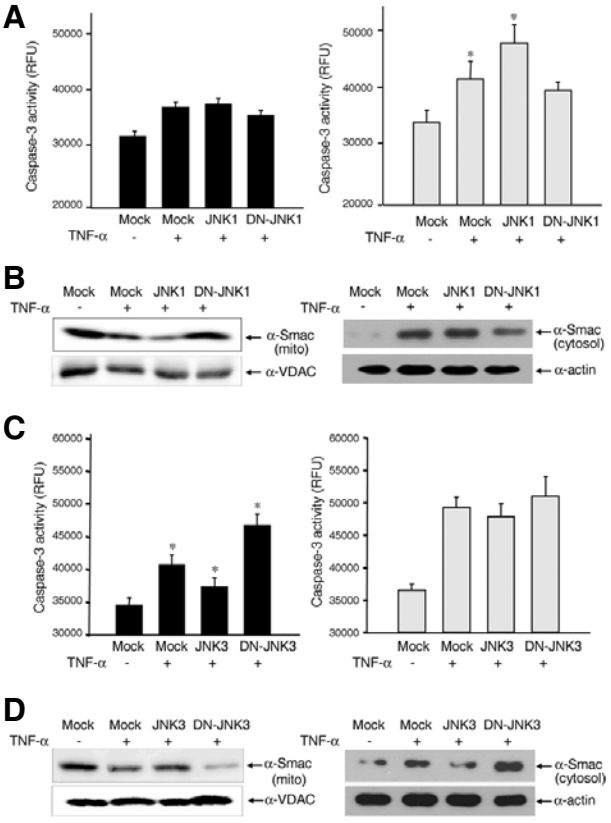

Fig. 4. JNK1 and JNK3 have different effects on caspase-3 activity and Smac release. (A) HeLa cells were transfected with expression vectors for Flag-JNK1 or Flag-DN-JNK1, or Mock as a control. Thirty-two h after transfection, cells were treated with TNF-α and cycloheximide for 5 h (left panel) or 7 h (right panel). The cells were harvested and caspase-3 activity was measured using Ac-DEVD-AMC as a fluorescent substrate. Data represents the means and standard deviation of at least three independent experiments. *P < 0.01 versus Mock (untreated). (B) HeLa cells were transfected with expression vectors for Flag-JNK1 or Flag-DN-JNK1, or Mock as a control, and then stimulated with TNF-α and cycloheximide for 7 h. Cells were harvested and subcellular fractions were prepared, as described in "Materials and Methods". Mitochondrial and cytosolic extracts were subjected to 12% SDS-PAGE and analyzed by Western blot using anti-Smac/DIABLO, anti-VDAC and anti-actin antibodies. (C) HeLa cells were transfected with expression vectors for Flag-JNK3 or Flag-DN-JNK3, or Mock as a control. Thirty-two h after transfection, cells were treated with TNF-α and cycloheximide for 5 h (left panel) or 7 h (right panel). Cells were harvested and caspase-3 activity was measured using Ac-DEVD-AMC as a fluorescent substrate. Data represents the means and standard deviation of at least three independent experiments. *P < 0.01 versus Mock (untreated). (D) HeLa cells were transfected with expression vectors for Flag-JNK3 or Flag-DN-JNK3, or Mock as a control, and then stimulated with TNF-α and cycloheximide for 5 h. Cells were harvested and subcellular fractionation was performed, as described in Materials and Methods. Mitochondrial and cytosolic extracts were subjected to 12% SDS-PAGE and analyzed by Western blot using anti-Smac/DIABLO, anti-VDAC and anti-actin antibodies.

JNK3 transfectants at the early time point (5 h) (Fig. 4D). These results suggested that JNK3 is involved in mediating cell survival at an early time point after exposure to TNF-α.

DISCUSSION

TNF is an important regulator of immune responses that also influences cell differentiation, survival and apoptosis. During TNF-mediated cell death, the activation of JNK can lead to opposite effects on the cell, depending on the timing and mechanism of activation. The rapid and transient activation of JNK mediated by TRAF2 Is associated with an anti-apoptotic effect (Limb et al., 2003), while the later and more sustained activation of JNK by ROS is associated with pro-necrotic effects (Sakon et al., 2003). It remains controversial whether JNK contributes to survival or apoptosis. JNK3 has been associated with neuronal death, and is responsible for prolonged JNK activation during TNF-induced cell death in the CNS (Jurewicz et

al., 2003). However, the function of JNK3 in non-neuronal cells is not well defined.

In this report, we demonstrated that JNK3 functions in an anti-apoptotic manner in immortalized cervical cancer cells. In cells that expressed a DN-JNK3, etoposide-induced apoptosis was enhanced as compared to mock- or DN-JNK1-expressing cells (Fig. 2). TNF-α-induced apoptosis was also enhanced in DN-JNK3-expressing cells as compared to JNK3-expressing cells. These results suggest that JNK3 prevents apoptosis in HeLa cells in response to etoposide or TNF-α.

During TNF-α-induced apoptosis in HeLa cells, JNK1 was activated in a biphasic manner, with a strong sustained and weak transient activation pattern. JNK3 activity and protein levels were induced early and strongly in a transient manner in response to TNF-α. Thus, the activation profiles and expression levels of JNK1 and JNK3 were quite different, providing further support for distinct roles for these two isoforms during apoptosis.

JNK1 and JNK2 display a strong degree of functional redundancy. However, neither JNK1 not JNK2 compensate for certain functions of JNK3. Mutations in *Jnk3* have been identified in 10 of 19 human brain tumors, which suggests that *Jnk3* is a candidate tumor suppressor gene (Kennedy and Davis, 2003; Mielke et al., 2000).

Recently, we reported that JNK3-mediated phosphorylation of Smac prevents apoptosis in HeLa cells following treatment with etoposide (Park et al., 2007). JNK3-mediated phosphorylation of Smac occurred during the early stages of etoposide-induced apoptosis. The results of the current study are in agreement with these previous results, and suggest that JNK3 functions at an early stage of TNF-α-induced apoptosis to promote cell survival.

We investigated caspase-3 activity and apoptosis in JNK1 and JNK3 transfectants at two different time points - an early and late time point. Smac release was enhanced in JNK1-expressing cells, and suppressed in JNK3-expressing cells. The perturbation of caspase-3 activity and Smac release by JNK3 occurred at an early time point (5 h), but not the later time point (7 h) in TNF-α-mediated apoptosis. These results indicate that JNK isoforms function in mitochondrial-dependent apoptosis, and differentially regulate apoptosis through the regulation of Smac release.

In summary, we have demonstrated that TNF-α-induced JNK3 activation is an early event in apoptosis, and functions in an anti-apoptotic manner through the suppression of Smac release. Thus, JNK1 and JNK3 differentially regulate TNF-α-mediated apoptosis in HeLa cells, and the anti-apoptotic role of JNK3 precedes the apoptotic role of JNK1.

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