c-Jun NH$_2$-terminal Kinase Activation Leads to a FADD-dependent but Fas Ligand-independent Cell Death in Jurkat T Cells*

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Persistent c-Jun NH$_2$-terminal kinase (JNK) activation induces cell death. Different mechanisms are ascribed to JNK-induced cell death. Most of the JNK-apoptosis studies employ stress stimuli known to activate kinases other than JNK. Here we used overexpression of mitogen-activated protein kinase kinase 7 (MKK7) to activate selectively JNK in T lymphoma Jurkat cells. Similar to that reported previously, Fas ligand (FasL) expression was up-regulated by JNK activation. Dominant negative-FADD and caspase-8 inhibitor benzyloxy-carbonyl-Ile-Glu-Thr-Asp effectively inhibited MKK7-induced cell death, supporting a major involvement of FADD cascade. However, MKK7-induced cell death was not prevented by antagonist antibody ZB4 and Fas-Fc, indicating that Fas-FasL interaction is minimally involved. Confocal microscopy revealed that persistent JNK activation led to clustering of Fas. Our results suggest that, in contrast to that reported previously, JNK alone-induced death in Jurkat cells is FADD-dependent but is not triggered by Fas-FasL interaction.

JNK$^1$ activation is always linked to cell death induced by stress. Apoptosis triggered by UV, γ-irradiation, and cytotoxic drugs is correlated with activation of JNK, and the cell death is prevented by inhibition of JNK activation (1–5). The pivotal role of JNK is further illustrated by JNK activation induced by active mitogen-activated protein kinase kinase 1 (MEKK1) (2, 6), active Cdc42 (7), and apoptosis signal-regulating kinase 1 (8) that either initiates the apoptotic process or potentiates cell death triggered by low dose stress stimuli (9). JNK activation is implicated in growth factor deprivation-induced cell death (10–12), in class I major histocompatibility complex ligation-triggered apoptosis (13), or possibly in anoisix (14). The critical role of JNK is also supported by the lack of apoptosis on hippocampal neurons in JNK3-deficient and in JNK1/JNK2 double knockout mice (15, 16).

The exact molecular mechanism how JNK induces cell death remains largely elusive. Different apoptotic molecules have also been attributed to JNK-triggered cell death. Activation of c-Jun by JNK seems to mediate part of the apoptotic events (17). MEKK1 or c-Jun induces FasL expression and the subsequent FasL-Fas interaction and cell death (6, 12, 18, 19). p53 and Bax may also mediate JNK-induced apoptosis following p75 neurotrophin receptor activation (11). Alternatively, apoptosis could be induced by translocation of JNK into mitochondria followed by phosphorylation and inactivation of Bcl-2 and Bcl-xL (20, 21). In addition, the UV-induced mitochondrial death pathway is abrogated in the absence of JNK, further supporting mitochondria as the target of JNK (22).

Most of JNK-inducing signals such as UV and cytotoxic drugs activate signals other than JNK. Even for the selective expression of MEKK1, Cdc42, or apoptosis signal-regulating kinase 1, activation of JNK is accompanied by stimulation of p38 and/or IκB kinase. In addition, the contribution of p38 to stress-activated apoptosis has been demonstrated (5, 10, 23, 24). In this study, we used transient expression of MKK7 to activate JNK in Jurkat T cells. MKK7 selectively activates JNK but not other kinases (25–29). We confirmed the previous notion that JNK activation leads to increased FasL expression in Jurkat cells. Blockage of FADD-initiated apoptotic pathway effectively prevented JNK-induced cell death. However, blockage of FasL-Fas interaction by antagonizing antibody or Fas-Fc did not affect MKK7-induced apoptosis, suggesting that FasL is minimally involved in JNK-mediated cell death. Our results clearly suggest that JNK induces apoptosis by a FADD-dependent but FasL-independent mechanism in Jurkat cells.

Experimental Procedures

Reagents—Jurkat cell (H6.2 clone) was a gift of Dr. Daniel Olive (INSERM U119, Marseille, France). Antibody against α-tubulin was obtained from Amersham Pharmacia Biotech. Anti-FasL antibody (C-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PE-conjugated anti-Fas antibody (DX2) and anti-FasL antibody (NOK-1) were purchased from e Bioscience (San Diego, CA). Anti-Fas antibodies CH11 and ZB4 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Caspase-8 inhibitor Z-IETD-fluoromethyl ketone was obtained from Calbiochem. Human Fas-Fc was purchased from R & D Systems (Minneapolis, MN).

Plasmids—Active MKK7a (S271D and T275D) (29) and active MKK3b were gifts of Dr. Jiahui Han (Scirpps Research Institute, La Jolla, CA). ΔMEKK1 and SEK-AL were gifts of Dr. Dennis Templeton (Case Western University, Cleveland, OH). pcDNAs-AU-FADD and DN-FADD were gifts of Dr. Vishva Dixit (Genentech, South San Francisco). FLIP plasmid was previously constructed (30).

Transfection—Jurkat T cells (1 x 10$^6$) were washed and resuspended in 0.6 ml of RPMI medium containing 1% glucose, 10% fetal calf serum, and 10–20 μg of plasmid DNA. The electroporation was performed in Bio-Rad Gene Pulser II at 260 mV and 975 microfarads. The cuvette was left on ice for 15 min, washed twice with phosphate-buffered saline, and then treated with 4 mM Na$_2$CO$_3$ for 20 min, and washed twice with phosphate-buffered saline.
and incubated for the indicated time for cell death and biochemical analysis.

**Protein Kinase Assay**—Jurkat T cells were transfected with pcDNA3, pcDNA-MKK7α, or ΔMEKK1 (each 5 μg), together with 3 μg of pGreen Lanten-1. After 24 h, cells were fixed with paraformaldehyde and proceeded with terminal dUTP nick-end labeling reaction. GFP-positive cells were then gated (R1) on FACSscan, and the incorporated biotin-dUTP in apoptotic cells was analyzed by Tri-Color-streptavidin (TC-SA) staining.

**Cell Death Measurement**—Apoptosis in bulk population was determined by propidium iodide (PI) staining. At the indicated times after treatment, cells were harvested and washed in phosphate-buffered saline twice and resuspended in hypotonic fluorochrome solution (50 μg/ml PI, 0.1% sodium citrate, 0.1% Triton X-100) (33). Cells were placed at 4 °C in the dark overnight, and DNA content was analyzed by FACSscan (Becton Dickinson, Mountain View, CA). The fraction of cells with sub-G1 DNA content was assessed using the CELLFIT program (Becton Dickinson). For apoptosis in cells transiently transfected with MKK7α or ΔMEKK1, green fluorescence protein expression vector pGreen Lanten-1 (Life Technologies, Inc.) was cotransfected. Cells were harvested at the indicated times, fixed with paraformaldehyde, and terminal dUTP nick-end labeling reaction was performed using FlowTACS kit (R & D Systems). The incorporated biotin-dUTP was labeled with Tri-Color-streptavidin (Caltag, Burlingame, CA). The green cells (GFP-positive) were then gated on FACSscan, and the fraction of cell stained with Tri-Color was quantitated. Alternatively, PI staining was also used to determined the fraction of subdiploid cells in GFP-positive population.

**RESULTS**

**Expression of Active MKK7α and MEKK1 Led to JNK-dependent Cell Death**—Jurkat cells were transfected with active MKK7α or MEKK1 by electroporation. We chose electroporation because the transfection efficiency was close to 30% as determined by cotransfection with GFP (Fig. 1A, R1). To assess
the cell death induced by JNK activation, the population expressing GFP was gated in fluorescence-activated cell sorter, and the fraction of apoptotic cells labeled with biotin-dUTP was quantitated (Fig. 1 A). Activation of JNK by MKK7α and MEKK1 in Jurkat cells led to 50% death 24 h after transfection (Fig. 1 B). We next examined whether the cell death observed was JNK-dependent. MKK7α and MEKK1 were equally effective in the JNK induction (Fig. 2 A). The specificity of MKK7α was further confirmed by its inability to activate p38 mitogen-activated protein kinase, in contrast to the effective induction of p38 by MEKK1 and MKK3 (Fig. 2 B). The activation of JNK by MEKK1 or MKK7α was prevented by cotransfection of SEK-AL (Fig. 2 A). The exact mechanism how SEK-AL prevents MKK7α-induced activation of JNK is not completely clear. Presumably, the binding of SEK-AL with JNK (34) would compete with the interaction of JNK with MKK7α. Inhibition of JNK activation by SEK-AL prevented MKK7α- or MEKK1-induced cell death (Fig. 2 C), indicating that the observed cell death is JNK-specific. Because MKK7α is a more specific activator of JNK, in the following experiments mainly the results of MKK7α transfection are shown. In all the criteria evaluated, MEKK1-induced apoptosis displayed an identical character.

MKK7α Expression-induced FasL Expression—Consistent with previous reports (6, 18), there was a significant increase of FasL expression after transfection of MKK7α and MEKK1 as determined by immunoblots (Fig. 3 A). Despite a similar degree of JNK activation (Fig. 2), the extent of FasL expression was higher for MEKK1 transfection than MKK7α transfection. A likely cause is because MEKK1 also activates NF-κB and p38 mitogen-activated protein kinase, and both contribute to activation of the FasL promoter (35–38). Despite the increase of total cellular FasL, there was little increase in the surface FasL.

**Fig. 2.** JNK-dependent cell death induced by active MKK7α. Jurkat cells were transfected by electroporation with 3 μg of pGreen Lanten-1, with active pcDNA3-MKK7α, AMEKK1, or MKK3 (each 5 μg), with or without 10 μg of SEK-AL. Cell extracts were prepared 18 h after transfection. **A,** JNK activities were determined by immunoprecipitation with anti-JNK1 (31) and phosphorylation of GST-c-Jun(1–79). **B,** p38 activities were determined by precipitation with anti-p38 (32) and phosphorylation of myelin basic protein (MBP). **C,** apoptosis was determined 24 h after transfection as described in Fig. 1. TC-SA, Tri-Color-streptavidin.
expression after MKK7α expression (Fig. 3B, dark curve). As a positive control, TPA/A23187 treatment significantly promoted the surface FasL expression (Fig. 3B, light curve). The expression of Fas is already high in Jurkat cells. Expression of active MEKK1 or MKK7α added little to the surface Fas expression (not shown).

MKK7α-induced Cell Death Was Blocked by DN-FADD and Caspase-8 Inhibitor IETD—Fas-mediated apoptotic pathway is initiated by recruitment of FADD, followed by cleavage and activation of caspase-8 (39). To examine whether MKK7α-induced cell death was indeed Fas-dependent, we used Fas-specific inhibitor DN-FADD, FLIP, and Z-IETD. DN-FADD competes with wild-type FADD (40, 41); FLIP specifically antagonizes Fas-dependent cell death at the stage of FADD and caspase-8 (42), and Z-IETD selectively inhibits caspase-8. Cotransfection with DN-FADD effectively inhibited MKK7α-induced apoptosis (Fig. 4). The expression of FLIP similarly inhibited MKK7α-induced apoptosis (not shown). The efficacy of caspase-8-specific inhibitor Z-IETD (50 μM) was first confirmed by blockage of CH11-induced apoptosis (not shown). The addition of Z-IETD 2 h after MKK7α transfection abrogated MKK7α-triggered apoptosis (Fig. 4). The inhibition of MKK7α-induced apoptosis by FLIP, DN-FADD, and Z-IETD supports the notion that FADD-mediated apoptotic pathway plays a major role in JNK-triggered cell death in Jurkat cells.

MKK7α-induced Cell Death Was Not Prevented by Fas-antagonizing Antibody and Fas-Fc—To determine the role of Fas engagement in MKK7α-induced cell death, the antagonistic anti-Fas antibody ZB4 was used. Preincubation with ZB4 (250 ng/ml) effectively suppressed CH11-induced apoptosis of Jurkat cells (Fig. 5A). In contrast, the extent of Jurkat cell death resulting from MKK7α overexpression was indistinguishable in the presence or absence of ZB4 (Fig. 5B). We also used soluble Fas-Fc fusion protein to block the interaction of Fas and FasL. Fas-Fc (200 ng/ml) prevented FasL-induced cell death (Fig. 5A), yet Fas-Fc failed to interfere with MKK7α-induced apoptosis in Jurkat cells (Fig. 5B). Because ΔMEKK1 induced higher expression of FasL (Fig. 3), we further examined whether ΔMEKK1-induced cell death could be inhibited by Fas-Fc or ZB4. Neither Fas-Fc nor ZB4 prevented apoptosis induced by ΔMEKK1 (not shown). The observations that Fas-Fc and ZB4 did not protect Jurkat cells from apoptosis suggest that the apoptosis induced by JNK is not mediated through Fas-FasL interaction.

MKK7-induced Fas Aggregation on Jurkat Cells—Stress stimuli such as UV, cycloheximide, cisplatin, etoposide, vincristine, and doxorubicin may induce cell death by triggering Fas clustering in a FasL-independent manner (43–46). We also examined the surface Fas distribution on a macroscopic level before and after JNK activation using confocal laser scanning microscope. Fas was evenly distributed on the surface of the untransfected Jurkat cells (Fig. 6A). Treatment with sFasL led to increased aggregation of Fas on the surface of Jurkat cells (Fig. 6B). For cells transfected with MKK7α, as those marked...
by GFP expression (Fig. 6C), there was a similar increased clustering of Fas on the surface of Jurkat cells as compared with the nearby untransfected cells (Fig. 6D). Therefore, constitutive MKK7α expression promotes the aggregation of the surface Fas.

**DISCUSSION**

JNK has been implicated as the major mediator of cell death induced by stress (1–3), yet the exact apoptotic mechanism triggered by JNK is not fully understood. In the present study, we used a JNK-specific activator MKK7α to induce JNK and the subsequent apoptosis. Because MKK7α selectively activates JNK (25–29), we were able to address death specifically activated by JNK in the absence of other signaling such as p38 (Fig. 2B). Our results illustrate that JNK activation induced FasL expression (Fig. 3). The requirement of Fas-FADD pathway is supported by the inhibition of MKK7-triggered cell death by DN-FADD, FLIP, and Z-IETD (Fig. 4). We have also demonstrated, likely for the first time, that JNK-induced cell death in Jurkat cells is FasL-independent (Fig. 5).

Our observations that Fas-Fc and ZB4 were unable to prevent MKK7α-induced cell death (Fig. 5) are in direct contradiction to the report of Faris *et al.* (6) that inducible expression of MEKK1 led to cell death which was prevented by soluble Fas and Fas antagonist antibody. We speculate the difference between their study and ours is likely due to the levels of Fas and FasL expression. In the study of Faris *et al.* (6), the inducible expression of MEKK1 in Jurkat led to an increase of surface FasL levels by 50-fold. This was accompanied by a 10-fold increase of surface Fas (see Fig. 5 in Ref. 6). With such high levels of Fas and FasL, Fas-FasL interaction would inevitably become the dominant process to trigger cell death. However, the levels of Fas and FasL induced by MEKK1 in their study
FIG. 5. MKK7α-induced cell death was not prevented by Fas-antagonizing antibody ZB4 and Fas-Fc. A, Jurkat cells were stimulated with CH11 antibody (100 ng/ml) or sFasL (100 ng/ml) in the absence or presence of ZB4 (250 ng/ml) or Fas-Fc (200 ng/ml) for 24 h. Cells were then stained with PI, and DNA content was analyzed by FACScan (Becton Dickinson). Percentage of cells with sub-G1 DNA content was assessed using CELLFIT program (Becton Dickinson). Each experiment was repeated at least twice. B, Jurkat cells were transfected with MKK7α and pGreen Lanten-1, and cell death was determined 24 h after transfection. When indicated, ZB4 (250 ng/ml) or Fas-Fc (500 ng/ml) was added right after transfection. Results were average of the three independent experiments.
are highly unphysiological, as judged from their report that there was a mere 50% increase of surface FasL with a 20% increase of surface Fas expression when Jurkat cells were activated with TPA/A23187 (6). In the present study, despite an induction of total FasL content, surface FasL levels in Jurkat cells were minimally altered by JNK activation (Fig. 3), and cell death proceeded in the absence of FasL binding. Fas-FasL interaction apparently is not essential for JNK-induced cell death in Jurkat cells. We have also repeated our observation in another T lymphoma EL4 (not shown) and reached an identical conclusion.

FADD-dependent but FasL-independent cell death is triggered by UV, cycloheximide, cisplatin, etoposide, vinblastine, and doxorubicin through induced clustering of Fas (43–46), leading to the association of FADD with Fas and the subsequent activation of caspase-8. Fas aggregation-induced cell death is suppressed by DN-FADD and Z-IETD (not shown), suggesting the presence of apoptotic pathway not mediated by caspase-8. JNK is known to induce apoptosis in Fas-independent manner by phosphorylation and inactivation of Bcl-2 and Bcl-xL (20, 21). Together with the results from the present study, persistent activation of JNK is capable of triggering apoptotic pathways initiated by both mitochondria (22) and death receptor. We speculate that the exact contribution from mitochondria and Fas pathway in JNK-mediated apoptosis would be determined by variables such as type of stress, type of cell, expression of Fas, and cellular sensitivity to Fas. Further characterization will help understand the exact molecular process triggered by JNK apoptotic signal in different cells.

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