The c-Jun N-Terminal Kinase Cascade Plays a Role in Stress-Induced Apoptosis in Jurkat Cells by Up-Regulating Fas Ligand Expression

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The c-Jun N-Terminal Kinase Cascade Plays a Role in Stress-Induced Apoptosis in Jurkat Cells by Up-Regulating Fas Ligand Expression

Mary Faris,* Niels Kokot,* Kevin Latinis,† Shailaja Kasibhatla,‡ Douglas R. Green,‡ Gary A. Koretzky,‡ and Andre Nel2*

T lymphocytes undergo apoptosis in response to cellular stress, including UV exposure and gamma irradiation. However, the mechanism by which stress stimuli induce apoptosis is not well understood. While stress stimuli induce the activation of the c-Jun N-terminal kinase (JNK) pathway, it is not clear whether the JNK cascade is activated as a result of cell death or whether the cascade participates in inducing apoptosis. Using a Jurkat T cell line transected with dominant active (DA)-mitogen-activated protein kinase kinase kinase (MEKK1) in a tetracycline-regulated expression system, we found that expression of DA-MEK1 results in the apoptosis of Jurkat cells in parallel with prolonged JNK activation. Moreover, DA-MEK1 induced Fas ligand (FasL) cell surface and mRNA expression, as well as FasL promoter activation. Interference with Fas/Fasl interaction prevented DA-MEK1-mediated apoptosis. In comparing the effect of different stress stimuli to DA-MEK1, we found that UV, gamma irradiation, and anisomycin prolonged JNK activation in parallel with FasL expression and onset of cell death. In addition, these stimuli also enhance cell surface expression of FasL. Interference with Fas/Fasl interactions inhibited anisomycin but not UV- or gamma irradiation-induced apoptosis. Our data show that while the JNK pathway contributes to stress-induced apoptosis in T lymphocytes by regulating FasL expression, not all stress stimuli use the same cell death pathway. The Journal of Immunology, 1998, 160: 134–144.

A poptotic or programmed cell death (PCD) is essential for maintenance of homeostasis in the immune system (1–4). Apoptotic cell death is induced in mature T cells upon ligation of the TCR (1, 2). In addition to this stimulus, T lymphocytes also undergo apoptosis when subjected to inflammatory cytokines, e.g., TNF-α, and environmental stress, e.g., exposure to gamma irradiation, hyperosmolarity, UV light, heat shock, lipid second messengers such as ceramide, and anticancer drugs such as etoposide (5–12). While TNF-α-mediated apoptosis depends on the requirement and activation of caspases that interact directly with the TNF type I receptor (TNFR1) (10, 11, 13–16), the mechanism by which other stress stimuli induce apoptosis is poorly defined. It has been demonstrated, however, that all of the above stimuli have the capacity to activate the c-Jun N-terminal kinase (JNK) cascade (6–9, 13–18), which, in turn, has been linked to the regulation of apoptosis (7–9, 17, 19–22). The original observation that apoptosis may be linked to the activation of the JNK cascade was made in PC12 pheochromocytoma cells during nerve growth factor withdrawal (20). Moreover, introduction of constitutively active mitogen-activated protein kinase kinase kinase (MEKK1), the principal MAP kinase in the JNK cascade (23), resulted in increased apoptosis in PC12 cells, while dominant interfering mutants of c-Jun, a downstream target of the JNK cascade, blocked apoptosis induction by nerve growth factor withdrawal (20). In gamma-irradiated T lymphocytes, an intimate temporal relationship between activation of the JNK cascade and induction of apoptosis has been demonstrated (7, 9). In addition, a dominant negative (DN) version of the Jun kinase kinase, SAP/extracellular signal-regulated kinase kinase SEK, or a dominant interfering version of c-Jun interfered with ceramide-induced apoptosis in other hematopoietic cell types (17). Recently, a novel mitogen-activated protein kinase (MAPK), ASK1, which activates the JNK cascade in epithelial cells, has been described and shown to induce apoptosis. In addition, DN-ASK1 interfered in the induction of apoptosis by TNF-α (22).

The role of JNK cascade in apoptosis is controversial, because induction of apoptosis by the TNFR1 or the Fas receptor is not hindered by disruption of the JNK cascade, e.g., by introduction of dominant interfering TRAF2 or JNK mutants (14, 24). Moreover, Johnson et al. have shown that dominant active (DA) MEKK1-mediated apoptosis in fibroblasts is not dependent on JNK or c-Jun activation (21). One view is that the activation of the JNK cascade by TNFR1 and Fas is a bystander event that follows rather than leads to apoptosis (14, 19). This is in agreement with the fact that...
a nucleus, and consequently activating protein-1 (AP-1)-induced gene expression, is not a critical requirement for apoptosis (14). What needs to be considered, however, is the possibility that the JNK cascade may regulate the expression of receptors, e.g., Fas and Fas ligand (FasL), that induce apoptosis. Taken together, the role of the JNK cascade in stress-induced apoptosis is controversial and needs to be clarified.

We have noticed that transient expression of DA-MEKK1 in Jurkat T cells leads to induction of apoptosis (not shown). More recently, we have begun to use the tetracycline (tet)-regulated vector system to control gene expression in lymphocytes (25–28). We therefore set out to determine whether inducible expression of DA-MEKK1 will lead to apoptosis in Jurkat cells, and whether this system is useful to clarify the mechanism of apoptosis induction by the JNK cascade. We demonstrate that constitutive activation of the JNK cascade by DA-MEKK1 induces apoptosis in Jurkat cells. This apoptotic event was accompanied by increased expression of Fas and FasL and could be inhibited by Fas-Fc protein. While different types of stress, including UV, gamma irradiation, anisomycin, and anti-Fas Ab, could induce apoptosis and prolonged JNK activation, Fas-Fc protein inhibited some but not all stress-induced apoptotic events. These results show that the JNK cascade, in association with other signaling pathways, plays an important role in stress-induced apoptosis.

Materials and Methods

Reagents

The anti-CD3 mAb, OKT3, was from Ortho Pharmaceuticals (Raritan, NJ) and anti-Fas mAb (CH11) was purchased from MRL (Camarillo, CA). Anti-CD28 (9.3) was a generous gift from Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA). Anti-ERK mAb was purchased from Zymed (San Francisco, CA), and horseradish peroxidase-conjugated protein A was purchased from Amersham (Arlington Heights, IL). The GST-c-Jun construct was generously provided by Dr. J. Woodgett (Ontario Cancer Institute, Ontario, Canada). G418, hygromycin, anisomycin, PMA, and ionomycin were purchased from Sigma (St. Louis, MO). The Cbz-Val-Ala-Asp-(Ome)-fluoromethyl ketone (Z-VAD) peptide was from Enzyme Systems (Dublin, CA). The tet-repressible system, including the pUHD15.1, pUHD10.3, pTPH, and pUHC13.3 vectors, was a kind gift of Dr. H. Bujard (Heidelberg, Germany) (25, 26). The pUHD15.1 plasmid encodes for the tetracycline-controlled transactivator (tTA), which is important role in stress-induced apoptosis.

Luciferase assays

A total of 10^5 Jurkat-Tα cells were transiently transfected with 10 μg of pUHC13.3 vector. Duplicate samples were pooled and grown in the presence of 0.1 μM dT, as indicated. Western blot analysis

Tet was withdrawn for 24 h from aliquots of 10^5 Jurkat-Tα cells transfected with DA-MEKK1. The cells were stimulated with 100 nM of PMA for 10 min, washed, and lysed as previously described (28). In all, 100 μg of cell lyses were separated by 10% SDS-PAGE and transferred to Immunoblot-P membranes (Millipore, Bedford, MA). The membranes were probed with 0.2 μg/ml of anti-ERK, followed by 1:3000 dilution of horseradish peroxidase-coupled protein A (29). The blots were developed with enhanced chemiluminescence according to the manufacturer’s instructions.

JNK assays

A total of 5 × 10^5 Jurkat cells were stimulated with 200 J/m2 UV, 3300 rad gamma irradiation, 1 μg/ml anisomycin, 100 ng/ml anti-Fas Ab, or a combination of 10 μg/ml anti-CD3 plus 2 μg/ml anti-CD28 for the indicated time period. The cells were lysed in 25 mM HEPES, pH 7.4, 50 μM β-glycerophosphate, 1 mM EDTA, 1% Triton X-100, 10 μM p-nitrophenylphosphate, 1 mM sodium orthovanadate, 2.5 mM MgCl2, 2 mM PMSF, 10 μg/ml leupeptin, and 2 μg/ml aprotinin. The supernatants were incubated with recombinant GST-c-Jun (1–79) bound to glutathione-coupled beads, and the complex washed extensively in lysis buffer. Kinase assays were performed as previously described (28). Fold increase in kinase activity was determined by densiometric analysis of the autoradiographic image as well as by subjecting gel slices to Cerenkov counting.

Measurement of apoptosis

The method of 7-aminopyrimidinyl D (7-AAD) staining was used to distinguish apoptotic from live cells (30, 31). Aliquots of 10^5 cells were incubated with 1 μg/ml of 7-AAD (Calbiochem, San Diego, CA) for 10 min at room temperature and analyzed by flow cytometry on a Becton Dickinson (Mountain View, CA) instrument using the analysis program Lysis II. This program distinguishes dead cells from cells undergoing apoptosis (31). To perform DNA laddering, genomic DNA was extracted by lysing the cells in lysis buffer containing 0.5 mg/ml of protease K and 100 μg/ml of RNase A, followed by phenol-chloroform purification (32, 33). DNA was separated on 2% agarose gel electrophoresis and the gels were viewed by UV transillumination. Tryptan blue staining was used to determine cell viability as determined by two independent observers. To determine the effect of the recombinant Fas-Fc protein (33) on induction of apoptosis by UV, gamma irradiation, and anisomycin, cells were incubated with 25 μg/ml of Fas-Fc for 15 min at room temperature before application of the stimulus as described above (33).

Fas and FasL expression

DA-MEKK1 cells were grown under off (tet(-)) or on (tet(+)) conditions for 24 h (28). For comparison, Jurkat-Tα cells were either left untreated or were stimulated with 100 nM PMA + 1 μg/ml ionomycin for 12 h. Immunostaining for Fas and FasL expression was performed by incubating the cells with the anti-Fas (CH11) or anti-Fasl (NOK1) mAb, followed by FITC-coupled anti-mouse Ig. The cells were analyzed by flow cytometry, using the Cell Quest program (Becton Dickinson).

RNA extraction and reverse transcriptase (RT)-PCR

Unstimulated and stimulated cells were washed and RNA was extracted using TRIzol according to the manufacturer’s recommendations (Life Technologies, Gaithersburg, MD). Reverse transcription was performed at 42°C in a reaction volume of 200 nM oligo(dT), 0.1 M DTT, 0.5 U RNAguard, 10 mM dNTP, 2 U MMTV, and 10 μg RNA (28). Semiquantitative PCR was conducted with Taq polymerase for 30 cycles consisting of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C. The primers used for β-actin amplification were: sense 5′-AATCTGGGACACACCTTCTACA and antisense 5′-CGAGTGAGCACACCCCTCTTATA, while the primers for FasL were 5′-CAACATCAAGGTCATGCTCTTAT and antisense 5′-AGATTGACCG. The PCR products were separated by 2% agarose gel electrophoresis and the gels were viewed by UV transillumination.
Table 1. Induction of apoptosis in Jurkat cells by stress stimuli

<table>
<thead>
<tr>
<th>Stress Stimuli</th>
<th>Percent Apoptotic Cells in the Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td>65.5 ± 2.3</td>
</tr>
<tr>
<td>Gamma irradiation</td>
<td>59.5 ± 3.1</td>
</tr>
<tr>
<td>Anisomycin</td>
<td>71.3 ± 2.6</td>
</tr>
<tr>
<td>anti-Fas</td>
<td>47.5 ± 4.2</td>
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</table>

*Jurkat tTA cells were stimulated with 200 J/m² UV, 3300 rad gamma irradiation, 1 µg/ml anisomycin, or 100 ng/ml anti-Fas mAb for 8 h. Cell death was determined by trypan blue exclusion. Duplicate counts were performed by two independent observers.

**Results**

**Stress-induced apoptosis in Jurkat cells occurs in parallel with JNK activation**

Chen et al. (7) and Chen, Meyer, and Tan (9) demonstrated an intimate temporal relationship between activation of the JNK cascade and induction of apoptosis in gamma-irradiated T cells. In addition to gamma irradiation, we found that exposure to UV, anisomycin, and anti-Fas Ab can induce apoptosis in Jurkat cells (Table I). Moreover, the same range of stimuli induced prolonged (>6 h) activation of the JNK cascade in Jurkat cells (Fig. 1A). Compared with unstimulated cells, the overall magnitude of stimulation by UV, gamma irradiation, and anisomycin amounted to 11-, 6-, and 10-fold, respectively. Transient activation of the JNK cascade by coligating the CD3 and CD28 receptors (Fig. 1B) did not lead the induction of PCD (Table II). Instead, CD28 coligation exerted an antiapoptotic effect on TCR-induced cell death (Table II). Please note that CD3 ligation in the absence of CD28 costimulation does not induce JNK activity (28). Taken together, these data suggest that the Jun kinase cascade and the kinetics of JNK activation may play an important role in stress-induced apoptosis.

Because the mechanism of action of this cascade in stress-induced apoptosis is unknown, we used DA-MEK1-K1 expression to study the role of the JNK cascade in apoptosis in Jurkat cells.

**Inducible expression of DA-MEK1 in Jurkat cells leads to constitutive and selective activation of the JNK cascade**

We established a Jurkat cell line that stably expresses the tet-regulated transcriptional activator protein, tTA. In the absence of tet, genes inserted downstream of a modified E. coli tet operator are induced (Fig. 2A) (25, 26). Subsequent transfection of Jurkat-tTA cells with a tet-suppressible vector (pUHD10–3) containing a DA-MEK1 (MEK1Δ) (10) insert yielded a cell line in which the JNK cascade was constitutively active in the absence, but not the presence, of tet (Fig. 2B). Compared with tetr(+) cells, unstimulated tetr(−) cells showed an eightfold increase in JNK activity (Fig. 2B, lanes 1 and 3). The specificity of this mutant kinase in the activation of the Jun kinase cascade was demonstrated by the failure of the expressed DA-MEK1 gene to induce ERK activation (Fig. 2C).

**DA-MEK1 induces apoptosis in parallel with JNK activation in Jurkat cells**

Jurkat-MEK1 cells, grown in the absence of tet, showed an increase in the number of trypan blue-positive cells in the population (Fig. 3A). Although there is an apparent lag period of about 12 h before the onset of cell death, this delay reflects the time required for DA-MEK1 expression and JNK activation (Fig. 3, A and B). On comparing the kinetics of JNK activation with the onset of cell death, we noticed that JNK activity began to increase from 6 h onwards and peaked at 48 h (Fig. 3, A and B). At that time point, 58% of cells in the population were dead, and continued to increase to yield 70% dead cells 72 h after tet withdrawal (Fig. 3, A and B). The decrease in JNK activity beyond the 48-h time period is due to the rapid decline in cell viability (Fig. 3B). Dying cells in the tetr(−) population were characterized by surface membrane blebbing, chromatin condensation, and appearance of apoptotic bodies, which collectively represent the features of apoptotic cell death (Fig. 4A) (19). To confirm that these cells indeed entered a program of PCD, we used 7-AAD staining and DNA laddering to demonstrate the characteristic pattern of DNA damage that accompanies apoptosis (Fig. 4, B and C) (13). Compared with tetr(+)
cells, DA-MEK1-expressing Jurkat cells showed a dramatic increase in 7-AAD uptake as determined by flow cytometry (Fig. 4B). The estimated rate of apoptosis in Jurkat-MEK1 cells was 40% after 48 h (Fig. 4B). Anti-Fas mAb was used as a positive control for apoptosis. Moreover, while expression of DA-MEK1 induced DNA fragmentation, cells maintained in the presence of tet failed to show DNA laddering (Fig. 4C). Taken together, these results show that prolonged DA-MEK1 expression and JNK activation induce apoptosis in Jurkat cells.

The induction of apoptosis by DA-MEK1 is dependent on Fas and FasL.

Possible roles for DA-MEK1 in induction of apoptosis include regulation of the cellular apoptosis threshold (34), as well as expression of receptors or ligands that induce apoptosis. Concerning a possible role of the JNK cascade in the expression of modulators of apoptosis, it has been shown that Ras, which acts upstream of the JNK cascade in T lymphocytes, regulates Bcl-2 and Bcl-xL expression in hemapoietic cells (35). These proteins heterodimerize with Bax, thereby inhibiting the formation of toxic Bax homodimers, which are required for apoptosis induction (36). Western blots that detect Bcl-2, Bcl-xL, and Bax (37) failed to show a change in the levels of these proteins in DA-MEK1-expressing Jurkat cells (not shown). We did, however, observe a dramatic increase in Fas and FasL expression in Jurkat DA-MEK1 cells. First, constitutive Fas expression in Jurkat cells, as determined by flow cytometry, was increased in tet(−) compared with tet(+) DA-MEK1 cells (Fig. 5A). DA-MEK1-induced Fas expression was more abundant than the increase obtained with PMA and ionomycin.

Table II. Failure of CD3 + CD28 coligation to induce apoptosis in Jurkat tTA cells

<table>
<thead>
<tr>
<th>Hours of Stimulation</th>
<th>Percent Apoptosis Following Stimulation with Anti-CD3</th>
<th>Percent Apoptosis Following Stimulation with Anti-CD3 + Anti-CD28</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.8 ± 1.8</td>
<td>4.3 ± 1.5</td>
</tr>
<tr>
<td>12</td>
<td>15.5 ± 2.3</td>
<td>3.4 ± 5.2</td>
</tr>
<tr>
<td>24</td>
<td>28.3 ± 4.6</td>
<td>3.4 ± 3.4</td>
</tr>
<tr>
<td>48</td>
<td>41.7 ± 2.5</td>
<td>6.1 ± 1.8</td>
</tr>
<tr>
<td>72</td>
<td>43.1 ± 1.8</td>
<td>7.9 ± 4.0</td>
</tr>
</tbody>
</table>

* Jurkat tTA cells were stimulated with 10 μg/ml anti-CD3 (OKT3) or a combination of 10 μg/ml anti-CD3 + 2 μg/ml anti-CD28 (9.3) for 24 h. Cell death was determined by trypan blue exclusion. Duplicate counts were performed by two independent observers.

![FIGURE 2](http://www.jimmunol.org/) Inducible expression of DA-MEK1 in Jurkat cells leads to the constitutive JNK activation. A, Luciferase assay to demonstrate tTA expression. Jurkat BMS2 cells were transfected with 10 μg of the pUHD15.1 plasmid, which encodes for the tTA. The cells were stably selected in 2 mg/ml of G418, and subclone D5 was transiently transfected with the reporter plasmid, pUHC13.3. The cells were grown in the presence (+) or absence (−) of 0.1 μg/ml of tet for 24 h, and assayed for luciferase activity. Fold increase was calculated using luciferase activity under tet(+) conditions as a baseline (1155 light units). B, In vitro kinase assay showing the constitutive activation of JNK by DA-MEK1. Jurkat-tTA cells were cotransfected with 30 μg of pUHD10.3 into which DA-MEK1 was subcloned and 5 μg of pTPH plasmid, which contains the hygromycin cassette. Following selection in 270 μg/ml of hygromycin, the cells were grown in the presence (tet+) or absence (tet−) of 0.1 μg/ml of tet for 24 h. Cells were either left untreated (lanes 1 and 3), or were stimulated for 10 min with 100 nM of PMA and 1 μg/ml of ionomycin at 37°C (lanes 2 and 4). Data shown in this experiment were reproduced three times. Fold increase in JNK activity was 6-fold for Tet(+) cells stimulated with PMA plus ionomycin (18,668 cpm), 8-fold for unstimulated DA-MEK1 Tet(−) cells (24984), and 15-fold in Tet(−) cells stimulated with PMA plus ionomycin (47,670 cpm) (lanes 2, 3, and 4). C, Western blot showing that DA-MEK1 expression does not activate the ERK cascade. DA-MEK1 cells were grown in the presence (+) or absence (−) of 0.1 μg/ml of tet for 24 h. The cells were lysed, and the cell lysates were analyzed by Western blotting with anti-ERK mAb. The hypomobility shift observed with anti-CD3 (OKT3) treatment (lanes 2 and 4) reflects ERK activation.
total cellular RNA that was reverse transcribed to cDNA, utilizing oligo(dT) primers and MMLV reverse transcriptase (38). Subsequent amplification with a set of FasL primers showed that while tet(+) cells lacked FasL message, DA-MEKK1-expressing cells showed abundant FasL mRNA (Fig. 5B). DA-MEKK1-induced FasL mRNA expression was more abundant than in PMA- plus ionomycin-treated cells, which is in accordance with flow cytometry data (Fig. 5, B and C). Taken together, these results show that DA-MEKK1 expression induces increased FasL mRNA and protein levels, which together with increased Fas expression, may be responsible for the induction of apoptosis in Jurkat cells.

To demonstrate that induction of apoptosis in DA-MEKK1-expressing Jurkat cells is Fasl mediated, we employed a recombinant Fas-Fc protein, which blocks the interaction between Fas and Fasl (33). Compared with the rate of cell death in DA-MEKK1-expressing cells, the inclusion of Fas-Fc in the culture medium reduced the rate of cell death rate by >80% [Fig. 5C]. These results indicate that interference with the Fas-FasL interaction prevents the induction of apoptosis by DA-MEKK (Fig. 5C).

To determine whether the stress stimuli depicted in Figure 1A can induce FasL expression, we used the flow cytometry approach shown in Figure 5, but could not obtain reliable data due to the rapid rate of cell death. We therefore employed a cysteine-protease inhibitor, Z-VAD, which interferes with the activation of CPP32 and ICE-like caspases (39-41), to determine whether we can prevent cell death long enough to determine FasL expression. Table III shows that inclusion of Z-VAD in the culture medium had a dramatic effect on the induction of apoptosis in Jurkat T cells by noxious stimuli and anti-Fas Ab. Overall, there was >80% inhibition of cell death in response to stimulation with UV, gamma irradiation, anisomycin, and anti-Fas mAb (Table III). These results indicate that induction of cell death by stimuli that also induce JNK activation in Jurkat cells. A, Expression of DA-MEKK1 induces cell death in parallel with JNK activation in Jurkat cells. A, Expression of DA-MEKK1 induces cell death of Jurkat cells in a time-dependent manner. DA-MEKK1 Jurkat cells were grown in the presence or absence of 0.1 μg/ml of tet for the indicated time period. Cell viability was measured by trypan blue exclusion. Duplicate counts were performed by two independent observers. Similar results were obtained in three separate experiments. B, In vitro JNK assay showing the time-dependent activation of JNK by DA-MEKK1. Duplicate aliquots of the cell populations shown above were lysed and assayed for JNK activity using the kinase assay described in Figure 1. No JNK activation was observed in DA-MEKK1 cells grown in the presence of tet (not shown).

The transcriptional activation of the Fas promoter is regulated by EA- and DN-MEKK1

To confirm that DA-MEKK1 and stress stimuli affect the FasL gene, we studied their effect on a FasL promoter reporter. We have previously described that treatment with anti-CD3 and PMA plus ionomycin induce activity of a luciferase reporter gene driven by 486 bp of genomic DNA immediately 5' of the Fasl translational start site (27). Transient transfection of this construct into Jurkat DA-MEKK1 cells showed 3.8- and 5.3-fold activation by anti-CD3 and PMA plus ionomycin, respectively, in tet(+) cells (Fig. 7A). After removal of tet, FasL-Luc activity increased to 7.9-fold without any additional stimulation (Fig. 7A). Treatment with anti-CD3 or PMA plus ionomycin did not significantly enhance further the response in tet(−) cells (Fig. 7A), showing that DA-MEKK1 expression alone resulted in maximum activation of the Fasl promoter.

In reverse experiments, we wished to determine whether DN-MEKK1 affects the activation of the FasL promoter. To perform this experiment, Jurkat-tTA cells were transiently transfected with DN-MEKK1 and FasL-Luc constructs before stimulation with UV, gamma irradiation, and anisomycin (Fig. 7B). Luciferase activity was compared in tet(+) and tet(−) cells. UV, gamma irradiation,
FIGURE 4. Inducible expression of DA-MEKK1 leads to the apoptosis of Jurkat cells. A, DA-MEKK1-expressing cells exhibit morphologic characteristics of apoptosis. DA-MEKK1 cells were grown in the presence (tet+) or absence (tet-) of tet for the indicated times. Morphologic characteristics were examined under phase contrast microscopy 48 h after the removal of tet (original magnification x200). B, FACS analysis showing that expression of DA-MEKK1 leads to intercalation of 7-AAD into cellular DNA. DA-MEKK1 Jurkat cells, grown in the presence or absence of tet, were stained with 1 μg/ml of 7-AAD 48 h after the withdrawal of tet, and analyzed by flow cytometry using the Lysis II program (Becton Dickinson). For comparison, Jurkat-tTA cells were treated with anti-Fas mAb (CH11) for 8 h. The percentages of apoptotic and live cells are included. These results were reproduced three times. C, DA-MEKK1 expression induces DNA laddering in Jurkat cells. Total DNA was extracted from DA-MEKK1 cells, separated by 2% agarose electrophoresis, and viewed by transillumination. Similar results were obtained in two additional experiments.
FIGURE 5. The induction of apoptosis by DA-MEK1 is dependent on Fas and FasL. A, Immunostaining showing enhanced Fas and FasL expression in cells expressing DA-MEK1. DA-MEK1 cells, grown under off (tet+) or on (tet-) conditions for 36 h (top panel), were stained with anti-Fas (CH11) or anti-FasL (NOK1) mAb, followed by FITC-coupled anti-mouse Ig, and analyzed by flow cytometry using the CellQuest program (Becton Dickinson). For comparison, Jurkat-tTA cells were either left untreated or were stimulated with 100 nM of PMA plus 1 μg/ml of ionomycin for 12 h (bottom panel) and analyzed as above. B, RT-PCR showing the enhanced expression of FasL mRNA in cells expressing DA-MEK1. DA-MEK1 cells grown in the presence of tet were either left unstimulated or were treated with 100 nM of PMA plus 1 μg/ml of ionomycin for 6 h (lanes 1 and 2). Lanes 3 and 4 represent DA-MEK1 cells from which tet was withdrawn for the indicated periods of time. RT-PCR analysis was performed using the primers for FasL and β-actin described in Materials and Methods. C, Inhibition of DA-MEK1-mediated apoptosis by the Fas-Fc fusion protein. DA-MEK1 cells were incubated in the presence or absence of 30 μg/ml of Fas-Fc and grown under (tet+) or (tet-) conditions for the indicated time periods. Cell viability was determined by trypan blue exclusion as described in Figure 3A.
and anisomycin induced 5.3-, 5.5-, and 4.1-fold increases in luciferase activity, respectively, in tet(+) cells. This response was reduced to 1.7-, 1.6-, and 1.1-fold, respectively, in DN-MEKK1-expressing cells (Fig. 7B). Taken together, these results show that MEKK1 plays an important role in the transcriptional activation of the FasL promoter.

Fas-Fc protein interferes in induction of apoptosis by anisomycin but not by UV and gamma irradiation

Induction of FasL expression constitutes a powerful mechanism by which cells regulate apoptosis (42). Since UV, gamma irradiation, and anisomycin were found to enhance the expression of FasL on the surface of Jurkat cells (Fig. 6), we determined whether interference with Fas binding to FasL will affect apoptosis induction by these stimuli. Addition of recombinant Fas-Fc proteins to the culture medium interfered with anisomycin-mediated apoptosis, reducing the rate of cell death by >60% (Fig. 8). As shown in Figure 5C, Fas-Fc inhibited DA-MEKK1-induced apoptosis by >80% (Fig. 8). In contrast, Fas-Fc had little effect on induction of cell death by UV or gamma irradiation (Fig. 8). These results indicate that while FasL expression is important for the induction of apoptosis by anisomycin, this ligand is not critical for induction of cell death by UV and gamma irradiation. This suggests that UV and gamma irradiation utilize alternative pathways to induce apoptosis.

Table III. Inhibition of apoptosis by the cysteine protease inhibitor Z-VAD

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Percent Apoptosis Following Stimulation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Absence of Z-VAD</td>
</tr>
<tr>
<td>Media</td>
<td>6.7 ± 1.3</td>
</tr>
<tr>
<td>UV</td>
<td>68.9 ± 9.1</td>
</tr>
<tr>
<td>Gamma irradiation</td>
<td>73.9 ± 5.5</td>
</tr>
<tr>
<td>Anisomycin</td>
<td>86.1 ± 5.9</td>
</tr>
<tr>
<td>Anti-Fas</td>
<td>55.1 ± 0.9</td>
</tr>
</tbody>
</table>

Jurkat cells were stimulated in the presence or absence of 30 μM Z-VAD. The percentage of apoptotic cells was determined 16 h later.

FIGURE 6. Immunostaining showing the induction of FasL expression by stress stimuli. Jurkat-tTA cells were stimulated for 14 h with 200 J/m2 of UV, 3300 rad of gamma irradiation, 1 μg/ml of anisomycin in the presence of 30 μM of Z-VAD. This prevents induction of apoptosis by these stimuli (see Table III). DA-MEKK1-expressing cells were used as a positive control. Immunostaining and FACS analysis were performed as described in Figure 5A.
In this paper, we show that exposure of Jurkat cells to UV, gamma irradiation, anisomycin, and anti-Fas Abs induce apoptosis in parallel with prolonged JNK activation (Fig. 1, A and B). We used tet-regulated expression of DA-MEKK1 in Jurkat cells to study the involvement of the JNK cascade in stress-induced apoptosis (Figs. 3, 4, and 5). We demonstrate that the increased rate of apoptosis in DA-MEKK1-expressing Jurkat cells is due to increased expression of FasL (Fig. 5, A and B). Fas-Fc protein interfered in the induction of apoptosis by DA-MEKK1 (Fig. 5C). While stress-induced apoptosis in response to anisomycin was accompanied by FasL expression, activation of the FasL promoter, and was Fas-Fc suppressible (Figs. 6–8), Fas-Fc did not interfere in apoptosis induction by UV treatment or gamma irradiation (Fig. 8). These data show that while some types of cellular stress utilize the JNK cascade to induce apoptosis, other types of stress lead to apoptosis in a JNK-independent fashion.

The tet-controlled gene expression system exhibits highly regulated and tight control of gene expression (25, 26). This system is ideal for performing in vivo biochemistry as demonstrated by the use of DA-MEKK1 to activate the JNK cascade and induce apoptosis in Jurkat cells (Fig. 3). While stress-induced apoptosis in response to anisomycin was accompanied by FasL expression, activation of the FasL promoter, and was Fas-Fc suppressible (Figs. 6–8), Fas-Fc did not interfere in apoptosis induction by UV treatment or gamma irradiation (Fig. 8). Data show that while some types of cellular stress utilize the JNK cascade to induce apoptosis, other types of stress lead to apoptosis in a JNK-independent fashion.

The importance of the kinetics of JNK activation in the induction of apoptosis in Jurkat cells is further underscored by the finding that transient JNK activation by CD3 plus CD28 coligation does not lead to apoptosis (Table II). These findings are in agreement with Chen et al. (7) and Chen, Meyer, and Tan (9), who demonstrated that stress-induced apoptosis in lymphocytes is accompanied by prolonged JNK activation. While the explanation for prolonged JNK activation in the induction of PCD is unknown, it is possible that this reflects regulation of AP-1 proteins and AP-1
response elements at a nuclear level. JNKs induce the transcriptional activation as well as increased expression of c-Jun (42). This may explain why dominant interfering c-Jun mutants are able to disrupt ceramide-induced apoptosis or apoptosis induced by growth factor withdrawal in PC12 cells (17, 20). JNKs also contribute to transcriptional activation of the ternary complex factor, Elk-1, which acts at the level of the c-fos promoter to increase expression of that gene (43, 44). It is interesting, therefore, that c-fos and c-jun proto-oncogenes have been found to play a role in PCD during growth factor deprivation in lymphoid cell lines (45). The effects of these proto-oncogenes on apoptosis could be opposed by c-fos and c-jun antisense oligonucleotides (45). Possible targets for AP-1-regulated apoptosis include genes that encode for proapoptotic proteins. An example is the Fas gene, which includes an AP-1 response element (TGAGTAA) at position −552 to −449 (46). This may explain the flow cytometry data that showed increased Fas expression in DA-MEKK1-expressing cells (Fig. 5A). We are currently looking at regulation of the Fas promoter by MAPK cascades. Another possible target for the JNK cascade is the FasL gene, since we have shown that both FasL message and protein can be induced in DA-MEKK1-expressing Jurkat cells (Fig. 5, A and B, and Fig. 6). Indeed, we found that DA-MEKK1 up-regulates the transcriptional activation of the FasL promoter in Jurkat cells (Fig. 7A). Although much remains to be learned about specific response elements in the FasL promoter, it is interesting that there is a consensus AP-1 element (TGACTCA) 10 to 16 nucleotides upstream from the start site (47). Further experimentation will be required to determine whether this element, or another sequence in the FasL promoter, is regulated by DA-MEKK1. Although it has been demonstrated that Fas induces JNK activation without requiring AP-1 response elements for induction of apoptosis (24), this situation differs from the experimental approach we describe. First, the induction of apoptosis by receptors that express death domains (e.g., Fas, TNFR1) can proceed independently of JNK activation and are typically rapidly executed events (14). Second, activation of the JNK cascade by Fas is a delayed event (24) that probably requires prior activation of caspases (48). Third, at the commencement of stress exposure, there are no ligands for death domain receptors expressed on the cell surface. This places JNK activation in a primary role from where it may induce expression of FasL (Fig. 6) or TNF-α (49) to commit cells to apoptosis. It is possible that in this setting, there is induction of AP-1 response elements that regulate these genes. It is also possible that MEKK1 regulates response elements other than AP-1, for instance, NF-κB binding sites. To this end, it has been shown that DA-MEKK1 induces activation of the IκB kinase, which leads to release of Rel proteins (50). We are in the process of studying the role of AP-1 and NF-κB response elements in the Fas promoter.

Our data, which show that DA-MEKK1 induces FasL expression in Jurkat cells, provide a novel explanation for the role of the JNK cascade in stress-induced apoptosis (Figs. 5 and 6). This finding is strengthened by data showing that Fas-Fc protein interferes with certain types of stress-induced apoptosis, e.g., anisomycin treatment (Fig. 5C). This finding implies that stress stimuli that use FasL expression utilize the same pathway for apoptosis induction as the TCR (1, 2). In activated lymphocytes, TCR can induce cell death through the expression of FasL. Since TCR itself does not activate the JNK cascade (28), it suggests that diverse signaling pathways lead to FasL expression. To this end, it has been shown that the TCR-associated protein tyrosine kinase, p56lck, can induce FasL message (38). Moreover, we have also shown recently that Ras is involved in the activation of the FasL promoter as well as the JNK cascade in Jurkat cells (28, 51). It is important to point out that not all types of stress that induce JNK activation use the Fas/FasL mechanism to induce apoptosis. For instance, while TNFR1- or Fas-mediated apoptosis was accompanied by prolonged JNK activation, interference in JNK activation by DN-TRAF2 or DN-JNKK, respectively, did not affect induction of apoptosis (14, 24). A possible explanation is that these receptors, through the recruitment of postreceptor proteins with death domains, lead to direct activation of caspase cascades (10, 11, 52, 53). In this setting, the JNK cascade is not required for receptor expression. The inability of Fas-Fc protein to interfere in UV- and gamma irradiation-induced apoptosis suggests the existence of additional stress-induced apoptosis pathways by which stress stimuli may induce apoptosis (Fig. 8). For instance, both UV and gamma irradiation are potent inducers of p38MAPK (54). At reduced doses of UV and gamma irradiation, apoptosis can be partly overcome with Fas-Fc protein. For instance, the addition of Fas-Fc protein to cells exposed to 1500 rad reduced the rate of apoptosis by 40%. It is interesting to note that when apoptosis is averted by a cysteine protease inhibitor, Z-VAD, FasL expression does occur in UV-exposed and gamma-irradiated cells (Fig. 6). We propose that prolonged JNK activation by stimuli that do not directly engage caspase cascades utilizes FasL expression as a back-up mechanism to eliminate activated or damaged T cells from the immune system. Under these circumstances a cell nucleus will be required for apoptosis. It is possible that activation of the JNK cascade by UV, gamma irradiation, TNF-α, and anti-Fas is the result rather than the cause of cell death (14, 19). It is noteworthy that ceramide release, which leads to JNK cascade activation (6, 17), follows the activation of caspases (48). This biphasic JNK response, which is depicted in Figure 1A, during anti-Fas stimulation is in keeping with this possibility.

It is clear from the foregoing that the role of the JNK cascade in apoptosis is complex and should consider the cell type as well as the type of cellular stress that is involved. Judged by the different effects of dominant interfering c-Jun mutants on apoptosis in PC12 cells, fibroblasts, and myeloid cells (17, 20, 21), it is clear that there are tissue-specific differences in the role of JNK in apoptosis. Within the immune system, the JNK cascade may play different roles in thymocytes and peripheral blood T cells. For instance, it has been demonstrated that sek−/− chimeric mice have normal numbers of mature T cells but fewer immature CD4−/CD8− thymocytes (55). It has therefore been suggested that Sek-1, a JNK, plays an antiapoptotic role in the thymus (55). Moreover, Sen et al. demonstrated that another MAP kinase cascade, p38MAPK, which is induced by cellular stress, plays a role in T cell survival in the thymus (56), whereas Graves et al. have shown that activation of the JNK and p38MAPK cascades by membrane Ig leads to apoptosis in B lymphocytes (57). As for the JNK cascade, p38MAPK may play different roles in apoptosis induction in different tissues. It is possible that the p38MAPK cascade may play an important role in the induction of apoptosis by gamma irradiation or UV exposure.

References

gamma radiation: duration of JNK activation may determine cell death and pro-