First publl. in: Molecular Cell ; 1 (1998), 4. - pp. 543-551

DNA Damaging Agents Induce Expression of Fas Ligand and Subsequent Apoptosis in T Lymphocytes via the Activation of NF-κB and AP-1

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Summary

Apoptosis induced by DNA damage and other stresses can proceed via expression of Fas ligand (FasL) and ligation of its receptor, Fas (CD95). We report that activation of the two transcription factors NF-κB and AP-1 is crucially involved in FasL expression induced by etoposide, teniposide, and UV irradiation. A nondegradable mutant of IκB blocked both FasL expression and apoptosis induced by DNA damage but not Fas ligation. These stimuli also induced the stress-activated kinase pathway (SAPK/JNK), which was required for the maximal induction of apoptosis. A 1.2 kb FasL promoter responded to DNA damage, as well as coexpression with p65 Rel or Fos/Jun. Mutations in the relevant NF-κB and AP-1 binding sites eliminated these responses. Thus, activation of NF-κB and AP-1 contributes to stress-induced apoptosis via the expression of FasL.

Introduction

Cells respond to cytotoxic stress and DNA damage either by cell cycle arrest and repair or by undergoing apoptotic cell death, depending on the cell type and the extent of damage. These responses depend in some cases upon the activation of p53 (Kuerbitz et al., 1992; Lowe et al., 1993) although other transcription factors have also been implicated. Such factors include c-Jun, c-Fos, ATF-2, and NF-κB, which are induced by ionizing or UV irradiation, tumor necrosis factor, heat shock, toxic drugs, and ceramide (Devary et al., 1992). Transactivation of c-Jun and ATF-2 is mediated by phosphorylation of the Ser/Thr residues in their activation domain by the proline-directed mitogen-activated protein (MAP) kinases, stress-activated protein kinases (SAPKs), or c-Jun N-terminal kinases (JNKs) (Kallunki et al., 1994; Kyriakis et al., 1994; Minden et al., 1994, 1995; Gupta et al., 1995; Livingstone, 1995).

Apoptosis induced by cytotoxic stressors has been shown to proceed through the activation of NF-κB and the SAPK/JNK pathway in some cases (Xia et al., 1995; Chen et al., 1996; Seimiya et al., 1997). Dominant-negative mutants of some of the elements of this pathway delayed or significantly inhibited apoptosis induced in some systems by a variety of cytotoxic agents or growth factor deprivation (Xia et al., 1995). In addition, ectopic expression of JNK can induce apoptosis (Chen et al., 1996). Likewise, daunorubicin, ara-C, and mitomycin C have been shown to activate NF-κB and induce apoptosis (Boland et al., 1997). These findings raise the possibility that transcription of one or more key "death genes" may be controlled by AP-1 and/or NF-κB transcription factors.

Apoptosis, per se, is not strictly dependent upon transcription (Martin et al., 1990). When apoptosis does require transcription, it is likely that this involves expression of proteins that signal the activation of the caspase proteases that orchestrate cell death. A relevant example is that of activation-induced apoptosis in T lymphocytes; apoptosis induced by activation of these cells is dependent on FasL transcription and expression (Alderson et al., 1995; Brunner et al., 1995, 1996; Dhein et al., 1995; Ju et al., 1995). FasL cross-links its receptor, Fas/CD95, and this results in apoptosis. This is one of the few examples where a requirement for gene transcription in apoptosis is understood.

Lymphocytes are especially sensitive to DNA damage–induced apoptosis, and it has been suggested that this sensitivity serves as a fail-safe mechanism against mutations that might allow host damage by defective cells of the immune system. Recent studies (Friesen et al., 1996) have provided evidence that apoptosis induced by some chemotherapeutic drugs in Jurkat T leukemia cells may proceed via the expression of FasL in a manner analogous to activation-induced apoptosis. Similarly, T cells from animals with defective expression of Fas or FasL function show a reduced sensitivity to apoptosis induced by γ irradiation (Reap et al., 1997). Thus, FasL expression appears to participate in DNA damage–induced apoptosis. We therefore examined whether cytotoxic stress–induced FasL in T lymphocytes might involve the activation of NF-κB and the SAPK/JNK pathway leading to the expression of FasL and death of the cell.

Results

DNA Damage–Induced Apoptosis Proceeds via Fas/FasL Interactions in T Cell Lines

To study DNA damage–induced Fas/FasL-mediated interactions, we used both a murine T cell hybridoma (2B4) in which activation-induced cell death (AICD) has been extensively characterized (Ashwell et al., 1987; Yang et al., 1995) and the Jurkat T leukemia cell line. We found that treatment of Jurkat T cells with the agents etoposide or teniposide, which damage DNA via their action on topoisomerase II (Kaufmann, 1989), or with ultraviolet B irradiation (UVB) induced apoptosis as well as significant levels of FasL mRNA, as detected by semiquantitative RT–PCR (see below).

We therefore examined the effects of blocking Fas/FasL interactions upon DNA damage–induced apoptosis in these cells. We had previously shown that a recombinant Fas-Fc chimeric protein (Fas-Fc) inhibits Fas/FasL interactions (Brunner et al., 1995). We found

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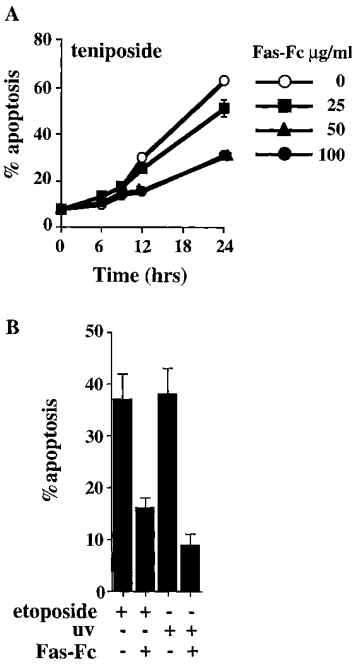

Figure 1. DNA Damage–Induced Apoptosis in T Cell Lines Proceeds via Fas/FasL Interactions

(A) Fas-Fc blocks DNA damage–induced cell death in Jurkat T cells. Jurkat T cells were treated with teniposide (5 μM) and cultured with the indicated concentration of Fas-Fc. The graph shows percent total apoptosis (Annexin V binding plus propidium iodide positive) at the indicated times. Similar results were obtained with etoposide treatment (not shown).

(B) Fas-Fc blocks DNA damage–induced cell death in 2B4 cells. The murine T cell hybridoma, 2B4, was treated with etoposide (10 μM) or UVB (60 s) and cultured with Fas-Fc (25 μg/ml). Cell death was determined after 16 hr by propidium iodide uptake on FACS.

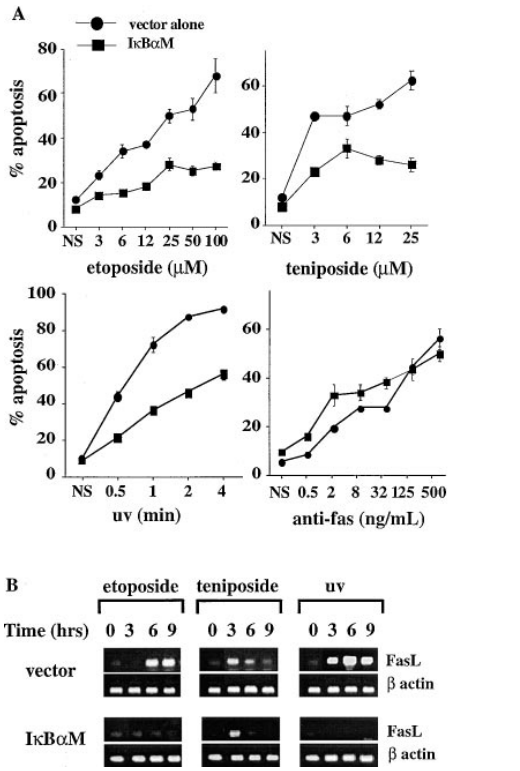

Figure 2. Inhibition of NF-κB Blocks DNA Damage–Induced Apoptosis and FasL Expression

(A) Jurkat T cells stably expressing IκBαM or the control vector were treated with the indicated DNA damaging agents or anti-Fas antibody, and cell death was determined after 12–16 hr by propidium iodide uptake on FACS.

(B) Jurkat T cells stably expressing IκBαM or the control vector were treated with etoposide (5 μM), teniposide (5 μM), or UVB (120 s), and FasL expression was determined by RT–PCR at the indicated times.

that Fas-Fc effectively blocked cell death induced by teniposide in Jurkat cells (Figure 1A). Similar results were obtained for etoposide and UVB (data not shown). We also observed this inhibition of stress-induced apoptosis in the murine T cell hybridoma 2B4 (Figure 1B) and in concanavalin A–activated murine T cell blasts (data not shown). Cell death was similarly blocked by neutralizing anti-Fas antibody (data not shown). Thus, competitive binding of either FasL (by Fas-Fc) or Fas (by anti-Fas) effectively inhibited death induced by DNA damaging agents in these cells.

DNA Damaged–Induced Fas/FasL-Mediated Apoptosis in Activated T Lymphocytes Proceeds via Activation of NF-κB

The signals that are induced by stress often activate the transcription factor NF-κB (Stein et al., 1993). Indeed, treatment with either of the topoisomerase inhibitors induced activity of an NF-κB-dependent reporter and not a mutated NF-κB reporter (data not shown). Previous studies by ourselves and others had suggested that NF-κB can act as an inhibitor of apoptosis induced by TNF but not by Fas (Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996), and we therefore sought to determine whether activation of NF-κB can either inhibit or promote stress-induced apoptosis, perhaps via effects on FasL expression.

To examine this possibility, we utilized an IκB mutant (IκBαM) that effectively blocks activation of NF-κB induced by either TNF or IL1 in a number of different cell lines, including Jurkat cells (Van Antwerp et al., 1996). As shown in Figure 2A, stable expression of IκBαM in Jurkat cells effectively inhibited apoptosis induced by etoposide, teniposide, or UV, but not by anti-Fas. This inhibition of apoptosis corresponded to an inhibition of stress-induced expression of FasL mRNA (Figure 2B). In contrast, and as previously shown (Van Antwerp et al., 1996), IκBαM overexpression increased the susceptibility to TNF-induced death (data not shown).

DNA Damage–Induced FasL Promoter Activation Is Dependent on the Activation of NF-κB

A simple, unifying explanation for the above observations is that DNA damage and related stress can induce the FasL promoter via the activation of NF-κB. To test this possibility, we employed a reporter construct in which luciferase gene expression is controlled by the 5′ flanking sequence (1.2 kb) from the human FasL gene. We cloned the 1.2 kb gene upstream of the initiation codon (Takahashi et al., 1994) of the human FasL promoter by polymerase chain reaction (PCR). The 1.2 kb

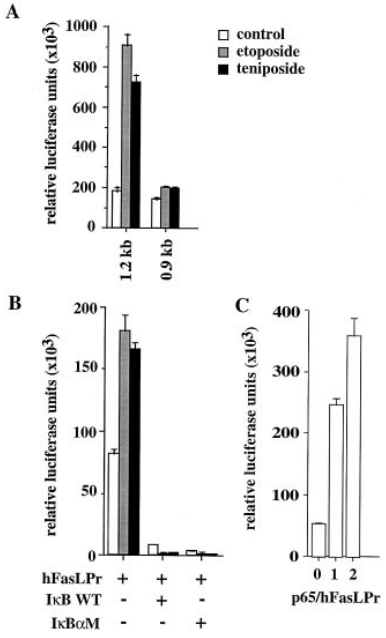

Figure 3. NF-κB Is Required for DNA Damage–Induced FasL Promoter Activation

(A) Jurkat T cells were transiently transfected with luciferase reporter constructs containing 1.2 or 0.9 kb of the FasL promoter. Cells were treated with etoposide (5 μM) or teniposide (5 μM), and reporter activity was determined after ~16 hr.

(B) Jurkat cells were transfected with the 1.2 kb FasL promoter reporter construct (20 μg) plus or minus expression constructs for IκB or IκBαM (20 μg). Cells were then treated with etoposide (5 μM) or teniposide (5 μM), and reporter activity was determined after 12–16 hr.

(C) The FasL promoter is induced by p65 Rel. Jurkat cells were transfected with the 1.2 kb FasL promoter construct plus an expression construct for p65 Rel at the indicated ratio. Reporter activity was determined after 12–16 hr.

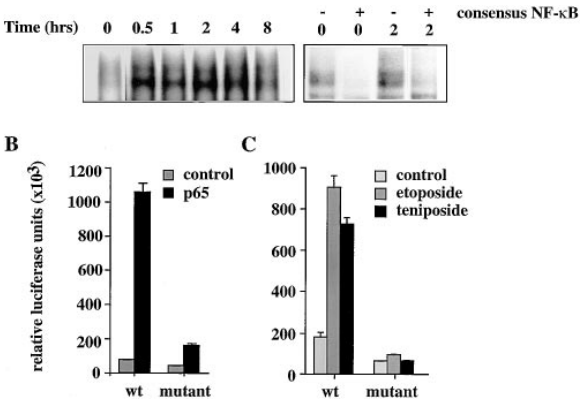

Figure 4. An NF-κB Binding Site in the FasL Promoter Is Required for DNA Damage–Induced Activation

(A) A putative NF-κB binding sequence in the FasL promoter binds stress-induced nuclear NF-κB. Nuclear extracts from Jurkat T cells treated with etoposide (10 μM) for the indicated times were employed in an EMSA with an oligonucleotide corresponding to a putative NF-κB binding sequence in the FasL promoter (GGGGACTT TCT). The mobility shift was competed by an unlabeled consensus NF-κB binding sequence (GGGGACTTTCC).

(B) Jurkat cells were transfected with the 1.2 kb FasL promoter construct, or a contruct containing a mutant NF-κB site (GGCGACTT TCT), plus an expression vector for p65 Rel. Reporter activity was determined after 12–16 hr.

(C) Jurkat cells were transfected with the 1.2 kb FasL promoter construct, or a construct containing the mutation described in (B), and cells were treated with etoposide (5 μM) or teniposide (5 μM). Reporter activity was determined after 12–16 hr.

FasL promoter contains putative binding sites for several transcription factors including AP-1, NF-κB, and NFAT. We observed that this construct showed consistent activation in Jurkat cells treated with a combination of phorbol esters and calcium ionophore as well as TCR ligation using anti-CD3 antibodies (data not shown), a stimulus that induces FasL promoter activation in T cells (Latinis et al., 1997). We then examined the ability of DNA damaging agents to induce activity of this promoter. As shown in Figure 3A, treatment of Jurkat cells with either etoposide or teniposide induced activation of the FasL promoter. This activation was not seen with a similar construct including only 0.9 kb of the FasL promoter. Putative binding sites for NF-κB and AP-1 were present in the region between 0.9 and 1.2 kb, and we speculated that these might participate in DNA damage–induced FasL expression and apoptosis (see below).

To test the effect of inhibition of NF-κB activation on stress-induced FasL promoter activity, we cotransfected Jurkat cells with the 1.2 kb FasL promoter reporter construct plus either wild-type IκB or IκBαM. As shown in Figure 3B, either form of IκB effectively blocked the activation of the FasL promoter by DNA damaging agents. These also inhibited basal activity of the promoter. It is therefore likely that NF-κB is required for stress-induced activation of the FasL promoter. Consistent with this idea, we found that coexpression of the p65 (RelA) subunit of NF-κB up-regulated FasL promoter activity (Figure 3C). These data further support a role for NF-κB in driving activation of the FasL promoter following DNA damage or related stress.

In an attempt to identify the potential NF-κB elements in the FasL promoter, we examined the promoter sequence and identified a putative atypical NF-κB binding site (GGGGACTTTCT) in the FasL promoter that is similar to the site found in the β2-microglobulin promoter (Israel et al., 1989). Following treatment with etoposide, we examined nuclear extracts for NF-κB binding activity by an electromobility shift assay (EMSA), using the putative NF-κB site from the FasL promoter. As shown in Figure 4A, etoposide treatment resulted in rapid activation of NF-κB binding activity. The binding to this site was competed by a consensus NF-κB motif (Lenardo and Baltimore, 1989) (Figure 4A) and not by an irrelevant oligonucleotide (data not shown).

We then performed site-directed mutagenesis to alter this site in the promoter and tested for the ability of the mutant FasL promoter to be induced upon treatment with DNA damaging agents. As shown in Figure 4B, this single nucleotide substitution abolished the ability of this promoter to be induced by coexpression of the p65 subunit of NF-κB. Further, the mutant FasL promoter was no longer induced by treatment with either etoposide or teniposide (Figure 4C). Together with the results described above, we conclude that NF-κB is a key mediator of stress-induced FasL expression.

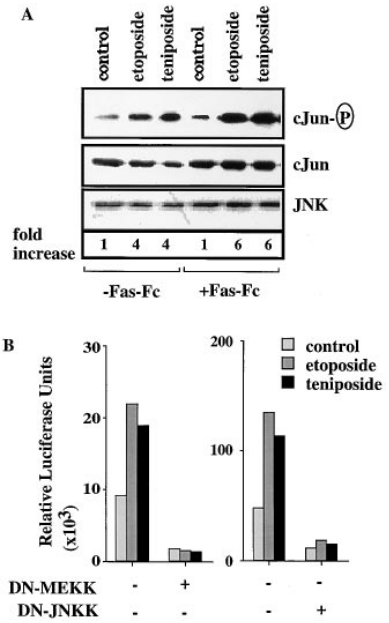

Figure 5. DNA Damage Induces JNK Activity Required for FasL Promoter Activation

(A) Effect of Fas-Fc on DNA damage–induced JNK activity. Jurkat cells were treated with 50 μM etoposide or teniposide or 80 J/m^2 UVB irradiation. After 45 min in the presence or absence of Fas-Fc (50 μg/ml), JNK activity was measured by the phosphorylation of GST-cJun in an in vitro kinase assay. Lysates from untreated cells were used as a negative control.

(B) Jurkat cells were transfected with hFasLPr (1.2 kb) and DN-MEKK1 (K432M) or DN-JNKK (K116R). Cells were treated with etoposide (5 μM) or teniposide (5 μM). Reporter activity was measured after 12–16 hr. Results shown are representative of three independent experiments.

Activation of the SAPK/JNK Pathway Is Required for DNA Damage–Induced FasL Expression and Apoptosis in Jurkat T Cells

To examine the role of the SAPK/JNK pathway in stress-induced FasL expression, we first confirmed that this pathway is engaged following such treatments. Treatment of Jurkat T cells with UVB, etoposide, or teniposide induced activation of JNK (data not shown), as described for other cell lines (Liu et al., 1996; Seimiya et al., 1997). In contrast, treatment with etoposide or teniposide had no effect on the activity of ERK (data not shown). The Fas-Fc chimeric protein that blocked apoptotic death had no effect on the increase in JNK activity (Figure 5A), suggesting that the activation of JNK occurs upstream of Fas/FasL interaction. Thus, as for UV and γ irradiation (Hibi, 1993; Derijard, 1994), exposure to topoisomerase inhibitors triggers the activation of the SAPK/JNK pathway, following which FasL is expressed.

To determine whether activation of the SAPK/JNK pathway participates in stress-induced apoptosis, we used a conditional gene expression system in which expression of a dominant-negative (DN) mutant of MEKK1 (K432M) is controlled by the presence or absence of tetracycline. MEKK1 acts as a mitogen-activated protein kinase kinase kinase (MAPKKK) in the JNK pathway (Minden et al., 1994). Expression of this DN-MEKK1 in Jurkat cells was shown to significantly inhibit JNK activity without affecting the ERK kinases (Faris et al., 1996). Under conditions in which DN-MEKK1 was expressed, apoptosis induced by either etoposide or teniposide was dramatically inhibited (71%–72% apoptosis was decreased to 25%–32% apoptosis, data not shown). In contrast, Fas-mediated apoptosis was unaffected by the expression of DN-MEKK1 (90% versus 96% apoptosis, data not shown). Thus, DNA damage–induced apoptosis in Jurkat T cells appears to be dependent upon the function of MEKK1 or other family members. Since we observe activation of JNK but not of ERK, it is likely that the effects of DN-MEKK1 represent a role for the JNK pathway in this form of apoptosis.

We then examined the effects of inhibition of this pathway on stress-induced FasL promoter activation. The 1.2 kb FasL promoter reporter construct described above was cotransfected with dominant-negative inhibitors of the SAPK/JNK pathway, and cells were treated with DNA damaging agents. As shown in Figure 5B, coexpression with either catalytically inactive DN-MEKK1 (K432M) or DN-JNKK (K116R) (Lin et al., 1995) inhibited stress-induced FasL promoter activity.

If the SAPK/JNK pathway is involved in activation of the FasL promoter, this most likely occurs via the activation of AP-1 (Karin, 1995). Indeed, we found that treatment of cells with either etoposide or teniposide effectively induced an AP-1 reporter construct (TRE-Luc) (Westwick et al., 1994) transfected into Jurkat T cells (data not shown). We therefore examined the FasL promoter sequence for possible AP-1 sites. One consensus site, TTAGTCAG (Lee et al., 1987), was identified. By EMSA, we found that this site bound nuclear AP-1 proteins that were competed by the consensus AP-1-binding sequence but not by a mutant sequence (Figure 6A). Based on these findings, we cotransfected cJun and/or cFos together with the 1.2 kb FasL promoter reporter construct as described above. As shown in Figure 6B, coexpression of Fos/Jun increased the promoter activity considerably. To determine whether this AP-1 site is important in stress-induced FasL expression, we then introduced mutations into this site in the FasL promoter and examined its effects. As shown in Figure 6C, mutation of the AP-1 site completely abrogated teniposide-induced FasL promoter activity, strongly suggesting that this site is essential for stress-induced activation of the promoter.

Discussion

DNA Damage–Induced Apoptosis Can Require Signal Transduction Events Leading to Gene Expression

In T cells and T cell lines, cytotoxic agents that damage DNA initiate a signaling pathway that involves the activation of NF-κB and AP-1, which in turn promote the apoptotic death of the cell. It is well established that AP-1 is induced via activation of the SAPK/JNK pathway (Kallunki et al., 1994; Kyriakis et al., 1994; Minden et al., 1994, 1995; Gupta et al., 1995; Livingstone, 1995), and there is also evidence that NF-κB can be induced as a

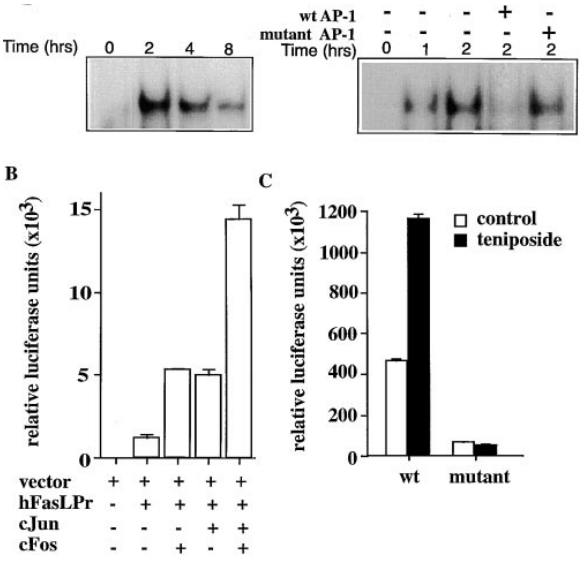

Figure 6. Activity of the SAPK/JNK Pathway Is Required for DNA Damage–Induced FasL Expression

(A) A putative AP-1 binding sequence in the FasL promoter binds stress-induced nuclear AP-1 proteins. Nuclear extracts from Jurkat T cells treated with etoposide (10 μM) for the indicated times were employed in an EMSA with an oligonucleotide corresponding to a putative AP-1 binding sequence in the FasL promoter (see text). The mobility shift was competed by an unlabeled consensus AP-1 binding sequence (TGACTCA) or a mutant sequence that does not bind AP-1 (TGACTGG).

(B) cFos and cJun activate the FasL promoter. Jurkat cells cotransfected with hFasLPr (1.2 kb) and expression vectors for cJun or cFos. Cells were harvested after 12–16 hr and assayed for reporter activity.

(C) Mutations in the AP-1 site were introduced into the 1.2 kb FasL promoter reporter construct. The wild-type (TTAGTCAG) or mutant (GATGTCAT) constructs were transfected into Jurkat cells, and the cells treated with teniposide (5 μM). Reporter activity was determined after 12–16 hr.

consequence of the SAPK/JNK pathway as well (Lee et al., 1997). Here, we have shown that activation of these two transcription factors is required for regulation of FasL expression, which in turn ligates Fas on the cell surface to trigger apoptosis. Thus, a pathway extends from DNA damage through the SAPK/JNK pathway, to activation of AP-1 and NF-κB, to expression of FasL and subsequent Fas-mediated apoptosis.

Recently, Faris and colleagues found that ectopic expression of a constitutively active MEKK leads to apoptosis and expression of FasL in Jurkat cells (Faris et al., 1998). This apoptosis was inhibited by blocking Fas/FasL interactions. Thus, activation of the SAPK/JNK pathway either by stress or by other means can result in activation of the FasL promoter via AP-1. Whether the activation of FasL expression and apoptosis by active MEKK is also dependent on NF-κB function is not known, but some reports suggest that NF-κB can be induced by this pathway (Lee et al., 1997). We suspect that stress-induced activation of AP-1 and NF-κB, leading to FasL expression and apoptosis, are linked via signaling pathways, with both elements required for optimal expression.

A number of cytotoxic stressors, including UV, ionizing radiation, chemotherapeutic drugs, and TNF, induce the activation of NF-κB (Rosette and Karin, 1996). NF-κB can also be activated via other pathways, many of which do not affect cell survival or death. Thus, how this transcription factor might contribute to the apoptotic process has remained elusive. Similarly, apoptosis induced in some cells by UV, ionizing radiation, and chemotherapeutic drugs can be inhibited by blocking the SAPK/JNK signaling pathway (Chen et al., 1996), although apoptosis induced by TNF may or may not depend on this pathway (Liu et al., 1996; Verheij et al., 1996), perhaps depending on the cell type being studied. This requirement of the SAPK/JNK pathway for some forms of apoptosis is most easily explained if the process of apoptosis requires transcription that is controlled by a factor, such as AP-1, ATF-2, or NF-κB, that is in turn regulated by the SAPK/JNK pathway. A similar case can be made for most other signaling events or molecules that have been implicated in apoptosis.

Transcription factors such as c-Myc (Evan et al., 1992; Amati et al., 1993; Hermeking and Eick, 1994), p53 (Kuerbitz et al., 1992; Fritsche et al., 1993), IRF-1 (Tanaka et al., 1994), nur77 (Liu et al., 1994; Woronicz et al., 1994; Yazdanbakhsh et al., 1995), and others are among the few molecules that have been identified as being required for some forms of apoptosis, and none of these are required for all forms. Thus, if we can determine which genes are regulated by these transcription factors en route to apoptosis, we can gain insight into how different pathways of apoptosis proceed. FasL is one example of such a gene.

Fas/FasL-Induced Apoptosis as One Pathway of Transcription-Dependent Apoptosis

Apoptosis induced by the ligation of Fas on the surface of a susceptible cell proceeds without a requirement for transcription (Yonehara et al., 1989; Itoh et al., 1991). However, expression of the components of this receptor/ligand pair is clearly under regulatory control such that the induction and expression of FasL in a cell that expresses Fas represents one role for transcription in apoptosis. This phenomenon of programmed cell death occurs upon activation of T cell lines or previously activated T lymphocytes (Anel et al., 1994; Alderson et al., 1995; Brunner et al., 1995, 1996; Dhein et al., 1995; Ju et al., 1995), as well as upon DNA damage in these cells (Friesen et al., 1996; Reap et al., 1997; our present work). Similarly, induction of Fas expression in cells that express FasL will also lead to apoptosis, and this has been shown to be a mechanism whereby IL1 induces apoptosis in thyrocytes (Giordano et al., 1997). A third way in which transcription could influence apoptosis in this system is via alteration of susceptibility to Fas-mediated apoptosis, perhaps through regulated transcription of the signal transduction components engaged upon ligation of Fas, or control of inhibitors of this signaling complex. Susceptibility to Fas-induced death is clearly regulated (Boldin et al., 1995, 1996; Muzio et al., 1996), and in one case, this susceptibility has been shown to be enhanced by expression of c-Myc (Hueber et al., 1997). Further, in the analogous process where apoptosis is induced by TNF, susceptibility has been shown to be inhibited by NF-κB (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996)

and enhanced by c-Myc (Janicke et al., 1996), as well as by Ras (Trent et al., 1996).

From this perspective, it is interesting to observe that activation of NF-κB is required for stress-induced expression of FasL (Figure 2). In the same cells, inhibition of NF-κB activation sensitized cells to apoptosis induced by TNF, although this did not affect susceptibility to Fas-mediated apoptosis in these cells (Van Antwerp et al., 1996), suggesting a differential role for NF-κB in apoptosis. It has been shown that a series of topoisomerase poisons (etoposide, camptothecin, ara-C) also activate NF-κB and induce apoptosis (Bessho, 1994; Piret and Piette, 1996), and our results suggest that this is important for the subsequent death of the cell.

We have identified an atypical NF-κB binding site, GGGGACTTTCT, in the FasL promoter that is similar to the site found in the β2 microglobulin promoter (Israel et al., 1989) and that we found to be responsible for the effects of NF-κB on the FasL promoter. Thus, it is possible that the contrasting effects of NF-κB activation on preventing TNF-induced apoptosis but promoting expression of FasL (and thus Fas-mediated apoptosis) represent a fail-safe mechanism to ensure lymphocyte death in response to cytotoxic stress. As mentioned in the Introduction, the importance of eliminating potentially damaged lymphocytes might require multiple mechanisms of apoptosis.

While we have focused on activated T lymphocytes and T cell lines in our studies, other cells may respond similarly to cytotoxic stress by undergoing FasL/Fas-mediated apoptosis, as has been observed in colon carcinoma lines (Houghton et al., 1997). Other examples may also exist. Radiation-induced apoptosis of lung endothelial cells is independent of p53, but dependent upon functional acidic sphingomyelinase (Santana et al., 1996). This enzyme is activated upon ligation of Fas (Cifone et al., 1993; Gulbins et al., 1995), and it has been suggested that its activity is important for Fas-mediated apoptosis in some cells. It will therefore be interesting to determine the role of stress-induced Fas/FasL interactions leading to apoptosis in these and other non-lymphoid cells.

It seems remarkable that any cell would use such a roundabout mechanism to mediate the apoptotic response to DNA damage. Even if the regulation of cell death via transcriptional control is desirable (allowing several levels of checks and balances on the system), why should the pathway to apoptosis require a de novo interaction of molecules on the cell surface?

Use of a cell surface receptor/ligand pair to activate caspases might provide an additional fail-safe mechanism to ensure that death occurs. Since cells that undergo cytotoxic stress are likely to be in the environment of other cells facing the same stress, a cell that might avoid undergoing apoptosis through failing to express sufficient amounts of functional FasL may nevertheless undergo apoptosis through interaction with a FasL-expressing neighbor. In this way, possible escape from DNA damage–induced apoptosis might be minimized.

This is not to say that this is the only pathway of cell death available to a T cell that has sustained DNA damage. We observed that T cell blasts from animals (*gld* mice) with functionally defective FasL expression (Takahashi et al., 1994) were more resistant to induction of apoptosis by low doses of etoposide than were those from wild-type controls (data not shown). However, at higher doses of etoposide (greater than 50 μM) no difference in the extent of apoptosis was seen (data not shown). Thus, in these T cells other mechanisms of DNA damage–induced apoptosis appear to be recruited to ensure that cell death occurs. This is consistent with the observation of others (Reap et al., 1997), who found that T cells from *gld* mice were relatively resistant to apoptosis triggered by γ irradiation.

We therefore do not propose that the expression of FasL and subsequent ligation of Fas is the only mechanism of DNA damage–induced apoptosis, nor the only one that may be regulated by NF-κB and the SAPK/JNK pathway. Nevertheless, our studies do indicate that this is one way that this signal transduction pathway can contribute to the apoptotic response, and suggest additional candidates for similar transcriptional activation. Further, we do not suggest that this pathway is exclusive to activated T cells, since other cell types are capable of expressing FasL and can undergo Fas-mediated apoptosis (Galle et al., 1995; Muller et al., 1997). The elucidation of this pathway not only gives us insights into the relationships between stress, signal transduction events, caspase activation, and apoptosis, but also provides a number of ways in which we can interfere with, or enhance, the response of some cells to cytotoxic stress.

Experimental Procedures

Cell Lines and Reagents

Human leukemic Jurkat cells (ATCC) and Jurkat cells stably transfected with SV40 large T antigen were used in the present study. The T cell hybridoma 2B4 has been described previously (Yang et al., 1995). All cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, and 100 U/ml each of penicillin and streptomycin (complete medium). Phorbol-myristate acetate (PMA) was purchased from Sigma (St. Louis, MO) and ionomycin from Calbiochem (La Jolla, CA). Mouse anti-human CD3 (OKT3) antibody and hamster anti-mouse CD3ε (145-2C11) was purified from the culture supernatant by protein A affinity chromatography. Anti-mouse CD95(Jo.2) was purchased from Pharmingen (San Diego, CA). Fas-Fc was produced by baculovirus expression system and purified using protein G affinity chromatography as described (Brunner et al., 1995).

Induction of Apoptosis

All experiments were performed in 96-well plates and in triplicate samples with cells resuspended at 1–5 × 10^6/ml in complete medium. For T cell receptor stimulation, 96-well plates were precoated with anti-CD3 antibody (2C11) in 50 mM Tris (pH 9.0). PMA and ionomycin were added at concentrations of 50 ng/ml and 0.5 μg/ml, respectively. UV radiation was used at 40–100 J/m^2. DNA damaging drugs etoposide (Sigma) and teniposide (Bristol-Myers Squibb) were used at 1–100 μM concentration. Anti-Fas antibody (CH-11; Kamiya Biomedical Lab) was used at 1–500 ng/ml concentration.

Assessment of Apoptosis

In most experiments, apoptosis was assessed by staining with Annexin V-FITC (BioWhittaker) plus propidium iodide and analyzed by FACS. In cotransfection experiments using tetracycline-repressable DN-MEKK (Faris et al., 1996), cells were grown in complete medium with or without 0.1 μg/ml tetracycline (Sigma, St. Louis, MO). After 40 hr, cells were activated with the DNA damaging drugs etoposide, teniposide, and anti-Fas antibody (Kamiya Biomedical Lab) for 6–8 hr, pelleted at 1–2 × 10^6/ml in PBS, and stained with Hoechst 33258 (Sigma) for 10 min at room temperature in the dark.

Cells were then analyzed by fluorescence microscopy (Nikon Optophoto) for apoptosis as assessed by nuclear condensation and fragmentation in the GFP-transfected cells.

FasL Reporter Constructs

A genomic clone of human FasL containing the putative promoter region was generously provided by Dr. S. Nagata. An approximately 8 kb HindIII fragment containing the 5′ promoter region was then subcloned into a eukaryotic expression vector HsLuc (Tillman et al., 1993) carrying a luciferase reporter gene downstream of the cloned fragment. Using overlapping primers and automated DNA sequencing (Core facility, SDSU, San Diego), we sequenced 2 kb upstream of the translational start site, which revealed potential binding sites for several transcriptional factors including the reported "housekeeping" promoter elements and NFAT binding sites (Takahashi et al., 1994; Latinis et al., 1997). Using PCR, we then obtained truncations of the FasL promoter region and subcloned these into the HsLuc vector 5′ of the luciferase reporter gene. The forward primers used for the 1.2 kb and the 0.9 kb gene were 5′-CCCAAGCTTCTGATATTTCAAA ACAGAATAGGAA-3′ primer 1 and 5′-CCCAAGCTTACTGGTTTGCA GCCTTCTGATCTA-3′ primer 2, respectively. The reverse primer 5′-ACGCGTCGACGGCAGCTGGTGAGTCAGGCCAGCC-3′ primer 3 was used for both amplifications. A 1.2 kb fragment and a 0.9 kb fragment of the hFasL promoter were subsequently used in the experiments described here. The mutations in the −1080 kB site were introduced using the Gene Editor kit (Promega) and in the −1048 AP-1 site using the Transformer site-directed mutagenesis kit (Clonetech), and both constructs were sequenced using automated sequencing (Applied Biosystems).

RT–PCR for FasL Expression

The expression of FasL was determined by reverse transcription (RT) of total RNA followed by reverse transcription–PCR analysis (RT–PCR). Approximatively 3×10^6 Jurkat cells were homogenized with 1 ml of Trizol reagent (GIBCO-BRL), and total RNA was isolated according to the manufacturer's protocol. cDNAs were synthesized by extension of (dT) primers with 200 U of SuperScript II reverse transcriptase (GIBCO) in a mixture containing 1 μg of total RNA digested by RNase-free DNase (2 U/μg of RNA) for 15 min at 37°C. PCR of the cDNA was performed in a final volume of 50 μl containing all four dNTPs, 2 mM $MgCl_2$, 2.5 U of AmpliTag (GIBCO), and each primer at 0.2 μM using the geneAmp 2400 PCR system (Perkin Elmer). The amplification cycles were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel after 30–40 cycles (471-bp human FasL fragment) or after 25–30 cycles (661-bp human β-actin) and visualized by ethidium bromide staining. Amplification of β-actin served as control for sample loading and integrity. The following primers were designed to discriminate between the amplification of cDNA and contaminating genomic cDNA: hFasL-Forward, TAA AACCGTTTGCTGGGGC; hFasL-Reverse, CTCAGCTCCTTTTTTTC AGGGG; β-actin-Forward, TGACGGGGTCACCCACACTGTGCC CA TCTA; and β-actin-Reverse, CTAGAAGCATTTGCGGTGGACG ATG GAGGG.

Expression Vectors and Transient Transfections

A conditional expression vector for DN-MEKK was generously provided by Drs. M. Faris and A. Nel (Faris et al., 1996). Expression vectors for Srα c-fos, c-jun, p65, IκB, and IκBαM were as previously described (Mercurio et al., 1993; DiDonato et al., 1996). Jurkat T cells containing stably transfected SV40 large T antigen were electroporated as described previously (Kasibhatla et al., 1993). Briefly, $1.5–2 \times 10^7$ cells were washed twice with serum-free RPMI 1640 medium, resuspended in 500 μl of the same medium and transferred to 4 mm gap electroporation cuvettes (BioRad). 20–60 μg of HsLuc FasL promoter alone or with Srα c-fos, c-jun, p65, IκB, or IκBαM expression vectors (Mercurio et al., 1993; DiDonato et al., 1996) were added to the cells and mixed well. Electroporation was carried out at 250 V and 960 μF in a Bio-Rad Gene Pulse II.

The 1.2 kb hFasL promoter construct was transfected with or without the coexpression vectors. pCMV β-galactosidase was used to normalize the transfection efficiencies in the various cotransfections. 40 hr posttransfection, cells were treated with the DNA damaging agents etoposide or teniposide and incubated for another 12–18 hr. Cells were harvested, washed three times with PBS, and lysed in 100 μl of the lysis buffer. Cell debris was removed by centrifugation, and the supernatant was used in the luciferase assay using a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

Electromobility Shift Assays

DNA binding reactions were carried out for 20 min at 4°C in a buffer containing 50 mM HEPES (pH 7.8), 20 mM $MgCl_2$, 0.5 mM EDTA, 20 mM spermidine, 500 μg/ml BSA, 10 mM DTT, 75% glycerol, and 10^4 counts per minute of labeled probe. The probes used were double-stranded synthetic oligonucleotides (Retrogen, San Diego, CA) representing AP-1 and NF-κB sites from the human FasL promoter: hFasL-NF-κB 5′-AAGCCTGGGCAACATAGAAAGTCCCCATCTGTA CAAAAA-3′, and hFasL AP-1 5′-AAAAAGAATAAATTAGTCAGG TGT AGTGACTTATGCCTC-3′. Each strand was labeled separately with T4 polynucleotide kinase (BRL) and [γ-^{32}P]ATP (5000 Ci/mmol), and the strands were then allowed to reanneal slowly. The samples were analyzed on a 4% nondenaturing acrylamide gel in 0.5% TBE. In some experiments, nuclear extracts from Jurkat cells were made as described (Rosette and Karin, 1995). Binding reactions contained 5–10 μg of nuclear extract, ^{32}P-labeled probe (25,000 cpm), and 2 μg of poly dI:dC in the binding buffer.

Acknowledgments

We are grateful to Dr. S. Nagata for providing us with the hFasL genomic DNA, Drs. M. Faris and A. Nel for a conditionally expressed DN-MEKK, and Dr. D. Van Antwerp for the IκBαM-expressing cell line. We would like to thank Dr. M. Karin for helpful discussions and Drs. Ruth Kluck, Helen Beere, and Tesu Lin for critical review of the manuscript. This work was supported by NIH grants GM 52735 and CA 69831 to D. R. G. This is publication #195 from LIAI.

Received November 24, 1997; revised December 29, 1997.

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GenBank Accession Number

The accession number for the sequence of the FasL promoter is AF044583.