NF-κB Signaling Pathway Governs TRAIL Gene Expression and Human T-cell Leukemia Virus-I Tax-induced T-cell Death*

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The Tax oncprotein encoded by human T-cell leukemia virus induces both T-cell activation and apoptosis. The mechanism by which Tax induces apoptosis has remained unclear. Using genetically manipulated T-cell lines, we demonstrate that Tax-induced T-cell death is dependent on NF-κB signaling. Tax fails to induce apoptosis in T cells lacking IkB kinase (IKKγ), an essential component of the NF-κB signaling pathway. This defect was rescued when the mutant cells were reconstituted with exogenous IKKγ. We further demonstrate that the Tax-induced T-cell death is mediated by TNF (tumor necrosis factor)-related apoptosis-inducing ligand (TRAIL), because this event can be effectively inhibited by a TRAIL-blocking antibody. Consistent with this functional aspect, Tax stimulates the expression of TRAIL mRNA. Finally, we provide genetic evidence demonstrating that the NF-κB signaling pathway is essential for TRAIL gene induction by both Tax and T-cell activation signals. These studies reveal a novel function of the NF-κB signaling pathway and suggest a key mechanism by which Tax induces T-cell death.

Human T-cell leukemia virus type I (HTLV-I) is an oncogenic retrovirus etiologically associated with the development of adult T-cell leukemia (1, 2). HTLV-I induces T-cell transformation both in vivo and in cell culture, and this transformation appears to involve a multistep process (3). At least in vitro, HTLV-infected T cells initially undergo proliferation and massive cell death, or apoptosis, followed by the appearance of immortalized cell clones (4) that appear to be equipped with anti-apoptotic factors (5). Over time, the immortalized T cells accumulate genetic abnormalities, leading to the generation of monoclonal transformed T-cell clones (3, 4). Emerging evidence suggests that both the apoptosis and immortalization of HTLV-infected T cells are mediated largely by the viral oncoprotein Tax (6–9).

Tax-induced T-cell immortalization involves aberrant induction of various growth-related cellular genes such as those encoding interleukin-2 (IL-2) and the α-subunit of the high affinity IL-2 receptor (10). Induction of many cellular genes by Tax is mediated through activation of NF-κB, a key regulator of genes involved in cell activation and growth (11, 12). Recent biochemical and genetic studies demonstrate that Tax activates NF-κB by stimulating the IkB kinase (IKK) (13–18), a multisubunit protein complex specifically phosphorylating the NF-κB inhibitory protein, IkBo (19). The IKK holoenzyme is composed of two catalytic subunits, IKKa and IKKβ, and a regulatory subunit, IKKy. Although lacking catalytic activity, IKKy is essential for IKK activation by both cellular stimuli and Tax (16, 17, 20). A Jurkat T-cell mutant lacking IKKy is defective in NF-κB activation by T-cell mitogens and Tax (17). This genetically manipulated T-cell system has been useful for a number of recent studies (17, 21, 22).

The mechanism by which Tax induces T-cell death remains largely unknown. A recent study suggests that this virus-specific event requires caspases (23), cysteine proteases that serve as common intracellular mediators of apoptosis (24). However, it is unclear whether Tax-mediated apoptosis involves any of the tumor necrosis factor (TNF) family of extracellular death regulators. This family includes TNF-α, Fas ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL), and other related proteins, all of which induce cell death by binding to their specific receptors on target cells (25). FasL plays a critical role in activation-induced cell death, which occurs in antigen-stimulated T cells (26). However, the FasL pathway is not important for Tax-mediated apoptosis induction (23). Here, we present genetic evidence suggesting that the Tax-induced T-cell death is largely mediated by TRAIL. Tax expression in Jurkat T cells results in potent induction of TRAIL gene expression, which is associated with massive cell death. This Tax-induced cell death can be inhibited with a TRAIL-blocking antibody. Interestingly, both the Tax-induced TRAIL gene expression and cell death require an intact NF-κB signaling pathway.

MATERIALS AND METHODS

Cell Lines—The parental Jurkat cell line and its derivatives, JM4.5.2, JM4.5.2-GFP, and JM4.5.2-IKKγ, have been described previously (17, 21). JM4.5.2 lacks expression of IKKγ and thus is defective in NF-κB activation by both cellular signals and Tax. The JM4.5.2-GFP and JM4.5.2-IKKγ are derivatives of JM4.5.2 stably reconstituted with green fluorescence protein (GFP) and IKKγ, respectively. The Molt-4 and SupT1 T-cell lines were provided by the AIDS Research and Reference Program of NIAID, National Institutes of Health. These cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 50 units/ml penicillin, and 50 μg of streptomycin. LAF is an IL-2-dependent HTLV-immortalized human T-cell line (kindly provided by Dr. G. Franchini (27)). CS166, HUT102, MT-2, and SLB-1 are IL-2-dependent HTLV-I transformed T-cell lines (14).

Reagents and Antibodies—Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma and used at a concentration of 10 ng/ml and 1 μM, respectively. The anti-TRAIL-blocking antibody

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‡ The abbreviations used are: HTLV-I, human T-cell leukemia virus type I; IL-2, interleukin-2; IKK, IkB kinase; FasL, Fas ligand; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; PMA, phorbol 12-myristate 13-acetate; GFP, green fluorescence protein; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcriptase-polymerase chain reaction; FACS, fluorescence-activated cell sorting.
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(RIK-2) was provided by Dr. H. Yagita (28). The anti-Fas-blocking antibody (M3) was provided by Immunex Corp. (29). The anti-Tax and anti-CD28 monoclonal antibodies were prepared from hybridoma 166B17-46-34 (provided by the AIDS Research and Reference Program, NIAID, National Institutes of Health) and 9.3 (provided by The Fred Hutchinson Cancer Research Center), respectively. FITC-conjugated annexin V (annexin-FITC) and propidium iodide were purchased from Roche Molecular Biochemicals.

**Retroviral Transduction**—Retroviral transduction was performed using the pCLXSN system provided by Dr. I. Verma (30). The cDNAs for GFP and Tax were cloned into the pCLXSN retroviral vector by standard methods, and the procedure for retrovirus production and infection was as previously described (21) except for the inclusion of vesicular stomatitis virus glycoprotein (provided by Dr. T. Friedmann (31)) in the packaging.

**RT-PCR and RNase Protection Analyses**—Total RNA was isolated from the various T-cell lines using TRI reagent (Molecular Research Center, Cincinnati, OH). RT-PCR analysis was performed as described (21) using the following primers: TRAIL, forward, GCTATGATGGTCCAGG, and reverse, GTCAATGCTCATCAAGTGCTC; GAPDH, forward, CTGATGACACACCTCATGGC; and reverse, CTGGCTT-CACACCCTCTGTATGC.

The RNase protection assays were performed using the RiboQuant Multi-probe RPA System (hAPO-3 template set, PharMingen) following the manufacturer’s instruction.

**Apoptosis Assay**—Apoptosis was analyzed based on the translocation of phosphatidylserine from the inner side of the plasma membrane to the outer layer during the early stages of apoptosis (32). Briefly, the indicated cells were stained with annexin V-FITC and propidium iodide, and the apoptotic cells were subjected to two-color FACS analysis (21). The early and late stage apoptotic cells are stained with annexin V and annexin V-propidium iodide, whereas the live cells are not stained (negative).

**Results**

**IKKγ Is Required for Tax-induced Apoptosis in Jurkat Cells**—To investigate the mechanism mediating Tax-induced T-cell death, we utilized a genetically manipulated T-cell system previously characterized in our laboratory (17, 21). This system includes an IKKγ-deficient mutant Jurkat T-cell line, JM4.5.2, the parental Jurkat cells, and JM4.5.2 cell derivatives reconstituted with GFP (JM4.5.2-GFP) or IKKγ (JM4.5.2-IKKγ). The JM4.5.2 and JM4.5.2-GFP cells are defective in NF-κB activation by both mitogens and Tax, whereas the parental Jurkat and JM4.5.2-IKKγ are competent in this cellular signaling pathway (17, 21). For efficient Tax expression, we infected the cells with a retroviral expression vector encoding Tax. Under these conditions, more than 95% of the cells were infected as assessed by a parallel infection using GFP-expressing retroviruses (data not shown). Consistent with a prior study (6), expression of Tax in the parental Jurkat T cells resulted in marked induction of cell death, whereas the mock infected cells (infected with retroviral vector lacking a cDNA) did not exhibit significant cell death beyond the background level detected in uninfected cells (Fig. 1A).

Three days post-infection, the cells were stained with annexin V-FITC and propidium iodide and subjected to FACS analysis. The cells singly stained with annexin V (lower right) and doubly stained with PI-annexin V (upper right) are early and late phase apoptotic cells, respectively. The double negative cells (lower left) are the surviving cells, the percentage of which in the total cell population is indicated below the graph. B, apoptosis analysis in parental (wild type (WT)) and mutant Jurkat cells. The cells were either not infected or infected with Tax followed by apoptosis assays as described in **A.** C, kinetics of Tax-induced apoptosis. D, RT-PCR analysis to monitor the expression of Tax (upper panel) and a control house-keeping gene, GAPDH (lower panel). RNA was prepared from cells at 4 days post-infection.

**Fig. 1.** The NF-κB signaling pathway is required for Tax-induced apoptosis in Jurkat cells. A, FACS profiles of apoptosis assays in Jurkat cells. Jurkat cells were either not infected, mock infected with empty pCLXSN vector (Vector), or infected with pCLXSN-Tax (Tax). Three days post-infection, the cells were stained with annexin V-FITC and propidium iodide and subjected to FACS analysis. The cells singly stained with annexin V (lower right) and doubly stained with PI-annexin V (upper right) are early and late phase apoptotic cells, respectively. The double negative cells (lower left) are the surviving cells, the percentage of which in the total cell population is indicated below the graph. B, apoptosis analysis in parental (wild type (WT)) and mutant Jurkat cells. The cells were either not infected or infected with Tax followed by apoptosis assays as described in A. C, kinetics of Tax-induced apoptosis. D, RT-PCR analysis to monitor the expression of Tax (upper panel) and a control house-keeping gene, GAPDH (lower panel). RNA was prepared from cells at 4 days post-infection.

**Tax-induced Apoptosis Is Blocked by Anti-TRAIL but Not Anti-Fas-blocking Antibodies**—To further investigate the mechanism of Tax-induced T-cell death, we examined the involvement of two important apoptosis-inducing molecules, FasL and TRAIL. Specific blocking antibodies for these two proteins were incubated with the Tax-expressing cells followed by measuring the effect of this treatment on Tax-mediated apoptosis. In agreement with a prior study (23), the Fas antibody did not generate an appreciable protective effect on Tax-induced cell death (Fig. 2, column 4). In contrast, the TRAIL-blocking antibody efficiently inhibited this virus-specific event (column 5). This finding suggests that TRAIL, but not FasL, is involved in Tax-induced apoptosis in Jurkat T cells.

**Tax Induces the Expression of TRAIL mRNA**—We then examined whether Tax induces TRAIL gene expression. RT-PCR
Inhibition of Tax-induced cell death by a TRAIL-blocking antibody. Jurkat T cells were either not infected (None), mock infected with pCLXSN vector (Vector), or infected with Tax (Tax). Some of the infected cells were incubated with the indicated blocking antibodies. At 3 days post-infection, the cells were subjected to apoptosis assays as described in Fig. 1. The percent of viable cells (double negative cells) in the total cell population is presented.

analysis revealed a strong induction of TRAIL mRNA by PMA and ionomycin (lane 2), mitogens that mimic the T-cell activation signals (Fig. 3A, upper panel). Interestingly, the TRAIL mRNA was also induced upon Tax expression. Parallel immunoblotting analysis readily detected the Tax protein in the infected cells (Fig. 3A, lower panel). This result, which was confirmed by the more quantitative RNAse protection assays (see Fig. 4), is in complete agreement with the finding that the TRAIL pathway is important for Tax-induced apoptosis (Fig. 2). We also examined TRAIL mRNA expression in a number of HTLV-transformed T cell lines (C8166, HUT102, LAF, MT2, and SLB-1) as well as control uninfected T-cell lines (Jurkat, Molt-4, and SupT1) using RNAse protection assays (Fig. 3B). Little or no TRAIL mRNA was detected in the HTLV-negative cell lines (lanes 1, 7, and 10). Consistent with the RT-PCR result, stimulation of Jurkat cells with PMA and ionomycin led to strong induction of TRAIL mRNA expression (Fig. 3B, middle panel, lane 2), which was further potentiated by cross-linking the CD28 costimulatory molecule using an agonistic antibody (anti-CD28) (lane 3). More importantly, TRAIL was constitutively expressed in all of the HTLV-transformed T cell lines except C8166 (Fig. 3B, middle panel, lanes 4, 5, 6, 8, and 9). Internal control assays revealed that the expression of caspase 8 was ubiquitous and not enhanced in HTLV-transformed cells (Fig. 3B, upper panel). Further, the total RNA amount in the different samples was similar, as demonstrated by the close to equal expression of a housekeeping gene (ribosomal L32). These results suggest that TRAIL gene expression is induced by HTLV-I infection and maintained in most of the virus-transformed cell clones.

Induction of TRAIL mRNA by Both Tax and T-cell Mitogens Requires the NF-κB Signaling Pathway—If TRAIL is the mediator of Tax-induced T-cell death, the expression of this death regulatory gene may also require NF-κB signaling. To examine this possibility, we performed RNAse protection assays to detect the expression of TRAIL mRNA in parental and IKK- deficient Jurkat T cells (Fig. 4). As expected, both mitogens and Tax stimulated TRAIL mRNA expression in the wild-type Jurkat cells (lanes 1–3). Remarkably, the TRAIL gene induction was almost completely blocked in the IKK-deficient JM4.5.2 cells (lanes 4–6). Further, this functional defect was largely rescued when the mutant cells were reconstituted with IKK (lanes 10–12) but not with GFP (lanes 7–9). Parallel immunoblotting assays readily detected Tax in all of the Tax-infected cell lines (data not shown). Together, these data clearly demonstrate that the NF-κB signaling pathway is essential for the inducible expression of the TRAIL gene.

In the same RNAse protection assay, we also examined the expression of caspase 8, FasL, and Fas, as well as TRAIL receptors, including the decoy receptors DcR1 and DcR3 and the killing receptors DR4 and DR5. As previously demonstrated (21), the mitogen-mediated FasL mRNA induction was not blocked by the IKKδ deficiency (Fig. 4, lanes 5 and 8), which is in sharp contrast to the induction of TRAIL. Additionally, we found that Tax is not a strong inducer of FasL, a result consistent with the dispensable role of the FasL/Fas pathway in Tax-induced cell death (see Fig. 2). Further, Jurkat cells exhibited constitutive expression of caspase 8, Fas, and DR5. Although the levels of Fas and DR5 were enhanced in mitogen-stimulated parental cells (lane 2), the inducible expression of these two genes appeared to be only moderately affected by the IKKδ deficiency.

DISCUSSION

The NF-κB signaling pathway plays an important role in apoptosis regulation (33). Although strong evidence implicates NF-κB as an antia apoptotic factor, there are studies suggesting that NF-κB may also function proapoptotically under certain situations (33, 34). The mechanism by which NF-κB promotes apoptosis remains controversial. Although overexpression of the RelA subunit of NF-κB induces FasL promoter activity, the NF-κB signaling pathway does not seem to be essential for the inducible expression of FasL in Jurkat T cells (21). In the current study, we have demonstrated that the NF-κB signaling plays a critical role in TRAIL gene induction by both T-cell mitogens and the Tax protein. This finding suggests a novel mechanism underlying the proapoptotic function of NF-κB.

Although Tax is best known for its T-cell activation function, several studies have demonstrated a potent apoptosis-inducing activity of this viral oncoprotein (6, 23, 35). It has been consis-
tently difficult to generate stable T-cell lines expressing high levels of Tax. Indeed, retrovirus-mediated expression of Tax in Jurkat T cells results in massive cell death within a few days in culture (Fig. 1). The Tax-induced Jurkat cell death can be blocked by a caspase inhibitor, zVAD (data not shown and Ref. 23), suggesting the involvement of common intracellular mediators of apoptosis. However, it has remained unclear how Tax initiates the apoptosis pathway. Tax has been shown to stimulate the promoter activity of FasL in reporter gene assays (35, 36) and enhance the level of FasL mRNA (23). However, Tax-induced T-cell death is largely insensitive to FasL-specific blocking antibodies (Fig. 2 and Ref. 23). This finding is somewhat surprising because Jurkat cells express high levels of Fas and are sensitive to Fas-mediated apoptosis (21). Our current study could explain these previous findings. Using the sensitive and specific RNase protection assays, we have shown that TRAIL, but not FasL, is strongly induced in Tax-expressing Jurkat cells (Fig. 4) as well as in various HTLV-transformed T-cell lines (Fig. 2B and data not shown). Indeed, a TRAIL-blocking antibody can effectively inhibit Tax-induced apoptosis in Jurkat cells. Our studies suggest that TRAIL is a major mediator of Tax-induced T-cell death.

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