Resistance to Apo2 Ligand (Apo2L)/Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)-Mediated Apoptosis and Constitutive Expression of Apo2L/TRAIL in Human T-Cell Leukemia Virus Type 1-Infected T-Cell Lines

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Adult T-cell leukemia (ATL), a CD4+ T-cell malignancy caused by human T-cell leukemia virus type 1 (HTLV-1), is difficult to cure, and novel treatments are urgently needed. Apo2 ligand (Apo2L; also tumor necrosis factor-related apoptosis-inducing ligand [TRAIL]) has been implicated in antitumor therapy. We found that HTLV-1-infected T-cell lines and primary ATL cells were more resistant to Apo2L-induced apoptosis than uninfected cells. Interestingly, HTLV-1-infected T-cell lines and primary ATL cells constitutively expressed Apo2L mRNA. Inducible expression of the viral oncoprotein Tax in a T-cell line up-regulated Apo2L mRNA. Analysis of the Apo2L promoter revealed that this gene is activated by Tax via the activation of NF-κB. The sensitivity to Apo2L was not correlated with expression levels of Apo2L receptors, intracellular regulators of apoptosis (FLICE-inhibitory protein and active Akt). NF-κB plays a crucial role in the pathogenesis and survival of ATL cells. The resistance to Apo2L-induced apoptosis was reversed by N-acetyl-l-leucinyl-l-leucinyl-l-norleucinal (LllL), an NF-κB inhibitor. LllL significantly induced the Apo2L receptors DR4 and DR5. Our results suggest that the constitutive activation of NF-κB is essential for Apo2L gene induction and protection against Apo2L-induced apoptosis and that suppression of NF-κB may be a useful adjunct in clinical use of Apo2L against ATL.

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent for adult T-cell leukemia (ATL), a malignancy of mature CD4+ T cells (18, 52, 69). HTLV-1-mediated T-cell transformation presumably arises from multiple oncogenic processes in which the virus induces chronic T-cell proliferation, resulting in accumulation of genetic defects and dysregulated growth of infected cells. Although the mechanisms of transformation and leukemogenesis are not yet fully elucidated, the viral protein Tax is thought to play a crucial role in these processes. Tax expression is sufficient to immortalize primary human CD4+ T cells and to transform rat fibroblast cell lines in vitro (1, 68), and it is capable of inducing tumors in transgenic mice (45). Apo2 ligand (Apo2L; also tumor necrosis factor [TNF]-related apoptosis-inducing ligand [TRAIL]) is a proapoptotic member of the TNF superfamily that also includes TNF-α and Fas ligand (16). Apo2L induces apoptosis of a variety of tumor cells (61, 64) by interacting with its cell surface receptors DR4/TRAIL receptor 1 (TRAILR1) (49) and DR5/TRAILR2 (63). DR4 and DR5 contain a cytoplasmic death domain. Oligomerization of the death domain in DR4 and DR5 recruits caspase 8 through cytoplasmic adaptor molecules and Fas-associated death domain protein (55) and activates the subsequent cascade of caspase proteases, resulting in apoptosis (2). On the other hand, DcR1/TRAILR3 (48, 58) and DcR2/TRAILR4 (9) lack a functional death domain and apoptosis-inducing capability. These “decoy receptors” compete with DR4 and DR5 for Apo2L binding. Thus, the types of Apo2L receptors used in the cells appear to be one of the key determinants of Apo2L-induced apoptosis sensitivity.

Induction of apoptosis of virus-infected cells may serve as a beneficial host defense mechanism to limit virus spread. Fas is a key player in activation-induced apoptosis of T lymphocytes, a host immunosurveillance system for elimination of virus-infected cells. Apo2L is also suggested to play an important role in virus-induced apoptosis. Conversely, it is advantageous for the virus to prolong the lives of infected cells in order to increase viral replication. Viruses have evolved a variety of strategies to dysregulate the normal cellular suicide program. On the other hand, in addition to the Fas ligand- and perforin-mediated pathways (22, 31, 36), it has recently been suggested that the Apo2L-mediated pathway also plays an important role in antitumor immunity. Apo2L is expressed on human CD4+ T-cell clones (26) and murine activated killer cells (27) and is involved in their cytotoxic activities against tumor target cells. ATL is difficult to cure using conventional therapies (66). The mechanism of chemoresistance of ATL cells is not yet well...
understood. Since dysregulated cell death has been causally implicated in malignant transformation, screening of ATL cells and HTLV-1-infected T-cell lines for the expression of apoptotic regulatory genes will not only contribute to our general understanding of leukemogenesis but could be useful for the design of new therapeutic approaches aimed at stimulating apoptotic responses in ATL cells. Apo2L may have antileukemic effects against ATL. However, the susceptibility of ATL cells and HTLV-1-infected T-cell lines to Apo2L-induced apoptosis is still unknown.

In the present study, by using HTLV-1-infected T-cell lines and primary ATL cells, we showed that Apo2L was not cytotoxic to these cells expressing surface DR5. We found that among various human T-cell lines, those infected with HTLV-1 preferentially coexpressed Apo2L, and the expression was at least in part mediated by Tax via the activation of NF-κB. In ATL cells, NF-κB activity is constitutively activated (42) and may play a crucial role in cell proliferation and transformation (43). We also show that activation of NF-κB appears to be a key transcription factor involved in resistance to Apo2L.

MATERIALS AND METHODS

Cell lines. The human T-cell lines Jurkat, MOLT-4, and CCRF-CEM; the HTLV-1-infected T-cell lines MT-2 (41), MT-4 (67), CEM (53), SLB-1 (30), HUT-102 (52), and MT-1 (40); and the acute promyelocytic leukemia NB4 cell line (32) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, Kans.). MT-2, MT-4, CEM, and SLB-1 are HTLV-1-transformed T-cell lines. MT-1 is a T-cell line of leukemic-cell origin established from ATL patients. The clonal origin of HUT-102 is unclear. JPY-9 and JPX-M (kindly provided by M. Nakamura, Tokyo Medical and Dental University, Tokyo, Japan) are subclones of Jurkat cells that express either Tax wild type or a nonfunctional Tax mutant, respectively, under the control of the metallothionein promoter (44, 47).

Clinical samples. Peripheral blood lymphocytes (PBLs) from healthy volunteers or patients with the acute (patients 1, 2, 4, and 5) or chronic (patients 3 and 6) type of ATL were analyzed. Mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). Each patient sample contained ~90% leukemic cells at the time of analysis. All samples were obtained after informed consent was received. PBLs of healthy volunteers were activated with phorbol myristate acetate (10 ng/ml) and recombinant human interleukin-2 (IL-2) (10 ng/ml).

Viability and apoptosis assays. The cytotoxic effects of Apo2L were examined by the use of cell proliferation reagent WST-8 (Wako Chemicals, Osaka, Japan). Cells were cultured for 5 h with WST-8; cells were cultured at a density of 10^6 cells/well, and PBLs were cultured at a density of 10^6 cells/well in the presence of various concentrations of recombinant human Apo2L (Super Killer TRAIL), purchased from Alexis Biochemicals (San Diego, Calif.). WST-8 (10 μl) was added for the last 4 h of incubation, and the absorbance at 450 nm was measured. The early apoptotic event in cell lines was examined by staining cells with propidium iodide and Annexin V conjugated to FITC (FACS Calibur; Becton Dickinson, San Jose, Calif.). The antigen defined by this antibody (7A6 antigen) is a 38-kDa protein localized to the membranes of mitochondria and is involved in the molecular cascade of apoptosis (57, 71).

Cell surface expression of Apo2L receptors. Surface expression of Apo2L receptors was analyzed by flow cytometry. NB4 cells served as a positive control for DR4, DR5, DR6, and DR3 expression. A total of 10^6 cells were incubated with 1 μg of biotinylated control mouse immunoglobulin G1 or monoclonal antibodies specific for DR4, DR5, DR6, and DR3 for 30 min. After being washed, the cells were incubated with phycoerythrin-conjugated streptavidin (Beckman Coulter, Marseille, France) for 30 min on ice and then analyzed by flow cytometry.

Plasmids and transfections. A series of expression vectors for Tax (pβMT-2 and Tax) and its mutants (Tax M22 and Tax 70S) were described previously (38, 59). IκBαΔN (5) and IκBαΔB (39) (kindly provided by D. W. Ballard, Vanderbilt University School of Medicine, Nashville, Tenn.) are deletion mutants of IκBα and IκBβ lacking the N-terminal 36 and 23 amino acids, respectively. The kinase-deficient K44M IκB kinase α (IKKα), K44A IKKB, and KK429/430AA NF-κB-inducing kinase (NIK) mutants have been described previously (11).

series of Apo2L promoter pGL3-luciferase reporter constructs described previously (15) were used to map the Tax-responsive regions. The internal deletion of the NF-κB site (ΔκB) was also created. Transfections were performed in Jurkat cells by electroporation using 5 μg of appropriate reporter and effector plasmids. To normalize transfection efficiencies, a thymidine kinase (TK) promoter-driven Renilla luciferase plasmid (pRL-TK; 2 μg; Promega, Madison, Wis.) was cotransfected as an internal control plasmid. The luciferase activities of total cell lysates were measured using the dual luciferase reporter assay system (Promega).

Reverse transcriptase (RT) PCR. RNA was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, Calif.). cDNA was synthesized from 1 μg of RNA using an RNA PCR kit (Takara Shuzo, Kyoto, Japan) with random primers. Thereafter, cDNA was amplified for 30 cycles for Apo2L, 35 cycles for DR4, DR5, DrR1, DrR2, FLICE-inhibitory protein (FLIPI), and FLIPs and 28 cycles for β-actin. The primer preparations for Apo2L, DR4, DR5, DrR1, DrR2, FLIPs, FLIP0, and β-actin were performed as described previously (50, 54). The cycling conditions were as follows: denaturing at 94°C for 30 s (for FLIPI, FLIP0, and β-actin) or for 40 s (for Apo2L, DR4, DR5, DrR1, and DrR2), annealing at 57°C for 60 s (for Apo2L) or at 60°C for 30 s (for FLIPI, FLIP0, and β-actin) or for 60 s (for DR4, DR5, DrR1, and DrR2), and extension at 72°C for 60 s (for Apo2L, DR4, DR5, DrR1, and DrR2) or for 90 s (for FLIPI, FLIP0, and β-actin).

Northern blot analysis. RNA was subjected to electrophoresis through a formaldehyde-agarose gel and transferred to a nylon filter. After transfer, the filters were prehybridized in 0.5 M sodium phosphate, 0.1% bovine serum albumin, 7% sodium dodecyl sulfate (SDS), 100 μg of salmon testis DNA/ml, and 100 μg of yeast RNA/ml for 2 h at 65°C. Hybridization was then carried out overnight in a prehybridization buffer containing the following: 50% formamide, 1× sodium citrate, 5× Denhardt's solution, 0.1% SDS, 0.1% sodium dodecyl sulfate, 0.1% Triton X-100, 100 μg of yeast RNA/ml, and 5× Denhardt's solution. The blots were washed with 2× sodium chloride, 0.1% sodium citrate, 50% formamide, 0.1% sodium dodecyl sulfate, and 0.1% sodium dodecyl sulfate at 60°C for 20 min. The blots were exposed to x-ray film for 16 h.

RESULTS

Sensitivity to Apo2L in T-cell lines and ATL cells. We studied the Apo2L sensitivities of a panel of T-cell lines. We performed an initial screen using the WST-8 assay to determine the sensitivities of the panel of T-cell lines to Apo2L (Fig. 1A and B). HTLV-1-negative T-cell lines (Jurkat, MOLT-4, and CCRF-CEM) were highly sensitive to Apo2L, with extensive killing at the lowest concentration of Apo2L. In contrast, HTLV-1-positive T-cell lines (CS/MJ, SLB-1, MT-2, MT-4, and B) were highly resistant to Apo2L. HTLV-1-negative T-cell lines (Jurkat, MOLT-4, and CCRF-CEM) were highly sensitive to Apo2L, with extensive killing at the lowest concentration of Apo2L. In contrast, HTLV-1-positive T-cell lines (CS/MJ, SLB-1, MT-2, MT-4, and B) were highly resistant to Apo2L.
HUT-102, and MT-1) showed resistance to Apo2L at the highest dose. We confirmed the results of the WST-8 assay by analysis of the 7A6 antigen, which is expressed on the mitochondrial membrane during apoptosis. HTLV-1-negative cell lines (Jurkat, MOLT-4, and CCRF-CEM) and HTLV-1-positive cell lines (C5/MJ and SLB-1) were treated with Apo2L, and the 7A6 expression was analyzed by flow cytometry. Consistent with the WST-8 assay, Apo2L induced apoptosis of Jurkat, MOLT-4, and CCRF-CEM cells, whereas very low levels of apoptotic cells were detected in C5/MJ and SLB-1 cells (Fig. 1C). Therefore, Apo2L-sensitive cell lines were HTLV-1 negative, whereas HTLV-1-positive T-cell lines were Apo2L resistant. We also evaluated the effects of Apo2L on freshly isolated ATL cells from six patients. ATL cells were resistant to Apo2L concentrations as high as 100 ng/ml (Fig. 1A). We determined Apo2L activities on resting and activated normal lymphocytes. Resting and activated PBLs from normal donors were incubated with Apo2L. Apo2L had no effect on resting PBLs. In contrast, Apo2L reduced the cell viability of normal activated PBLs (Fig. 1A, right). In agreement with our findings, previous studies (37) indicated that PBLs become sensitive to the action of Apo2L following stimulation with IL-2. Because ATL cells phenotypically resemble activated T cells, HTLV-1 might in some way contribute to Apo2L resistance.

Apo2L expression in HTLV-1-infected T-cell lines and primary ATL cells. Although HTLV-1-infected T-cell lines and primary ATL cells secrete large amounts of transforming growth factor β (TGF-β), infected cells are resistant to the growth-inhibitory activity of transforming growth factor β (19, 29). A similar close linkage between resistance to the growth-inhibitory factor and its high expression might be observed in the case of Apo2L. Therefore, we investigated the expression of Apo2L in HTLV-1-infected T-cell lines and primary ATL cells. The results of RT-PCR analysis of Apo2L mRNA levels in several T-cell lines are shown in Fig. 2A. Apo2L transcripts (536 bp) were detected in all six HTLV-1-infected T-cell lines but were hardly detected in the uninfected T-cell lines. These results demonstrate that Apo2L is selectively expressed in HTLV-1-infected T-cell lines. To assess the relevance of our findings, we analyzed the expression of Apo2L mRNA in primary blood cells from ATL patients. As shown in Fig. 2B, Apo2L mRNA was expressed at high levels in leukemic cells of ATL patients. In contrast, Apo2L mRNA was hardly detected in lymphocytes of healthy volunteers.

**Tax induces *Apo2L* gene expression.** Because Tax is known to induce a number of cellular genes, we next examined whether Tax itself caused up-regulation of *Apo2L* gene expression. For this purpose, we used JPX-9, which stably carries a Tax expression plasmid, pMAXneo, in which expression of Tax...
is inducible by the addition of CdCl₂ (44, 47). The level of expression of Tax mRNA in these cells was determined by Northern blot analysis, and expression of the Apo2L gene was assayed by RT-PCR. As shown in Fig. 2C, the addition of CdCl₂ to the culture medium of JPX-9 cells induced the expression of Tax within 5 h, and it persisted until 72 h after treatment. pMAXneo contains an intron with splice donor and acceptor sites in the Tax coding region (47). The two hybridized bands correspond to spliced and unspliced Tax mRNAs, respectively. A concomitant increase in the expression of Apo2L within 10 h of treatment with CdCl₂ was observed in JPX-9 cells. The induction of Apo2L could not be attributed to CdCl₂ treatment, because Apo2L expression was not induced in JPX/M cells expressing a nonfunctional Tax mutant protein after treatment with CdCl₂ (data not shown). These results indicate that Tax itself is capable of augmenting the expression of the Apo2L gene in Jurkat cells.

**Tax transactivation of Apo2L promoter.** We investigated whether Tax-mediated up-regulation of Apo2L gene expression could directly enhance the activity of its promoter. Jurkat cells were transfected with a reporter gene construct containing the −1056 nucleotides of the Apo2L upstream regulatory sequences (ApoP−1056). Coexpression of Tax caused a 17-fold increase in the activity of this Apo2L-driven reporter construct (Fig. 3A). Tax can stimulate transcription through distinct transcription factors, including cyclic-AMP-responsive element binding protein and NF-κB. Next, through the use of two additional Tax mutants that selectively retain the ability to activate the cyclic-AMP-responsive element within the HTLV-1 long terminal repeat (M22) or NF-κB (703) (38, 59), we determined the pathway required for activation of the Apo2L promoter by Tax. Whereas wild-type Tax and the 703 mutant, which could activate NF-κB, increased Apo2L-driven reporter gene activity, no significant activation of the reporter was observed with the M22 mutant (Fig. 3A). Therefore, NF-κB activation contributes to activation of the Apo2L promoter by Tax.

We further examined whether Tax-mediated activation of Apo2L gene expression involves signal transduction components in NF-κB activation. Activation of NF-κB requires phosphorylation of two conserved serine residues of IkBα (Ser-32 and Ser-36) and IkBβ (Ser-19 and Ser-23) within their N-terminal domains (12). Phosphorylation leads to ubiquitination and the 26S proteasome-mediated degradation of IkBs, thereby releasing NF-κB from the complex to translocate to the nucleus and activate genes (12). A high-molecular-weight complex, IKK complex, which is composed of two catalytic IkB kinase subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ, phosphorylates IkBs (23). Previous studies indicated that members of the mitogen-activated protein kinase kinase kinase protein kinase family mediate physiologic activation of IKK (70). These kinases include NIK (65) and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (33). We examined whether Tax-mediated transactivation of Apo2L gene expression involves signal transduction components in NF-κB activation. Dominant-interfering mutants of
IκBα and IκBβ and kinase-deficient mutants of IKKα, IKKβ, and NIK were tested for the ability to inhibit Tax-mediated activation of the Apo2L-driven reporter gene. Expression of these various inhibitory mutants inhibited the activation of the Apo2L promoter by Tax (Fig. 3B). These data demonstrate that signaling components, NIK and IKK, involved in the activation of NF-κB are necessary for Tax transactivation of the Apo2L promoter.

Previous experiments showed that two potential NF-κB binding sites, κB2 (located between −384 and −375) and κB1 (located between −264 and −255), contained within the Apo2L promoter are important in up-regulation following T-cell activation (4). To assess the importance of the NF-κB sites within the Apo2L promoter, luciferase constructs containing sequential deletions of the Apo2L promoter were transfected into Jurkat cells, together with Tax expression plasmid. As shown in Fig. 3C, deletion of sequences down to position −126 did not diminish promoter activation, while further deletion to position −33 significantly decreased Tax-induced Apo2L activation. These data suggest that the Apo2L promoter containing −126 and −33 is required for Tax-induced Apo2L activation. Since the −126/−33 region lacks previously characterized NF-κB binding sites, κB1 and κB2, the DNA sequence was analyzed. The sequence analysis revealed the presence of overlapping NF-κB and SP1 binding sites in the sequence between −75 and −65 in the Apo2L promoter (Fig. 4A). To test the role of this site in Tax-mediated induction of Apo2L gene transactivation, we generated a −1056/+86 Apo2L promoter-reporter construct bearing an internal deletion of this site (ApoP/1056ΔκB). The effect of Tax on activation of wild-type and ΔκB Apo2L promoters was analyzed in Jurkat cells transfected with a Tax expression plasmid. Consistent with previous findings, our experiments showed that Tax expression resulted in an ∼34-fold increase in Apo2L promoter activation (Fig. 4B). Furthermore, deletion of the −74/−65 region resulted in ∼66% reduction of promoter activation, confirming the importance of this site in Apo2L gene expression. These observations indicate that the −74/−65 region is involved in Tax-mediated activation of Apo2L.

Tax induces binding of NF-κB family proteins to NF-κB element of Apo2L promoter. Because deletion analyses of the Apo2L promoter indicated that Tax activated transcription through the −74/−65 region, it was important to identify the nuclear factors that bind to this site. JPX-9 cells were incubated with CdCl₂ and 72 h after challenge, nuclear protein extracts were prepared and analyzed for DNA binding activity. As shown in Fig. 4C, a complex formed with the oligonucleotide probe corresponding to the Apo2L sequence between −81 and −51 was induced in JPX-9 cells within 72 h after the addition of CdCl₂. This binding activity was reduced by the
addition of cold probe or the typical NF-κB sequence of the IL-2Rα enhancer, but not by an oligonucleotide containing a mutated NF-κB sequence, an SP1 sequence of the MMP-9 promoter, or an AP-1 sequence of the IL-8 promoter (Fig. 4C, lanes 3 to 7). Next, we characterized the Tax-induced complexes identified by the probe. These complexes were supershifted or reduced by the addition of anti-p50, anti-p65, or anti-c-Rel antibody, but not by anti-SP1 antibody (Fig. 4C, lanes 8 to 13), suggesting that Tax-induced Apo2L NF-κB binding activity is composed of p50, p65, and c-Rel. These results indicate that Tax induces Apo2L gene expression, at least in part through the induced binding of p50, p65, and c-Rel to an NF-κB site that spans from −74 to −65 in the Apo2L promoter region.

Binding of NF-κB family proteins to Tax-responsive elements within Apo2L upstream regulatory sequences in HTLV-1-infected T-cell lines. Since we showed that HTLV-1-infected T-cell lines express significantly more Apo2L mRNA than do uninfected T-cell lines, we sought to determine whether HTLV-1-infected T-cell lines better exhibited NF-κB DNA binding activity. Using the oligonucleotide probe corresponding to the Apo2L sequence between −81 and −51 in EMSA, we observed clear shifted bands when these probes were incubated with nuclear extracts from the HTLV-1-infected T-cell lines C5/MJ and SLB-1 but not with nuclear extracts from uninfected cells (Fig. 5A). These shifted complexes were specific to the NF-κB sequence, because complex formations were reduced by the addition of excess cold probe and the typical NF-κB sequence of the IL-2Rα enhancer but not by an oligonucleotide containing a mutated NF-κB sequence, an SP1 sequence of the IL-2Rα enhancer, or an AP-1 sequence (Fig. 5B, lanes 2 to 6). Furthermore, to identify factors bound to the probe, we performed a gel shift assay using antibodies. Supershifts were seen with anti-p50, anti-p65, and anti-c-Rel antibodies, illustrating that the complex with probe contains p50 and p65 subunits of NF-κB and c-Rel, as shown in Fig. 4C. These results indicate that the increased activity of NF-κB binding plays an important role in the observed activation of the Apo2L gene in HTLV-1-infected T-cell lines. Thus, HTLV-1 infection induces Apo2L gene expression, at least in part, through the induced binding of p50, p65, and c-Rel NF-κB family members to an NF-κB site that spans from −74 to −65 in the Apo2L promoter, and this effect is at least in part Tax dependent.

Expression of Apo2L receptors on T-cell lines. To investigate whether the sensitivity of T-cell lines to Apo2L depends on the expression of Apo2L receptors, we used RT-PCR (Fig. 6A). The expression of DR5 mRNA was detected in all of the cell lines studied. The expression of DR4 mRNA was detected at relatively low levels in Jurkat cells, but not in the other cell lines. The RT-PCR products of DcR2 were detected in all cell lines C5/MJ and SLB-1 but not with nuclear extracts from uninfected cells.
lines except SLB-1 cells, whereas the expression of DcR1 mRNA was not detectable in any tested cell lines. The localization of death receptors is regulated in some cell systems (73), and therefore, we used flow cytometry to determine their cell surface expression on cell lines (Fig. 6C). Consistent with the RT-PCR analysis, DR5 expression was detected in all cell lines, whereas DR4 was expressed at relatively low levels on Jurkat cells. DcR1 and DcR2 were not detected on any tested cell lines, although all cell lines except SLB-1 cells had the RNA transcript of the \( \text{DcR2} \) gene. The degree and pattern of proapoptotic (DR4 and DR5) and antiapoptotic (DcR1 and DcR2) receptors did not correlate with the sensitivity to Apo2L-induced cell death in T-cell lines.

Expression of intracellular regulators of apoptosis in Apo2L-sensitive and -resistant T-cell lines. Fas-associated death domain protein and caspase 8 are obligate molecules for transducing apoptotic signals via death receptors, and FLIP has been shown to interfere with the formation of Apo2L receptor signaling complexes (20). We therefore analyzed the expression of FLIP mRNA to determine its potential role in determining sensitivity to Apo2L-induced cell death in T-cell lines.

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Inhibition of NF-κB reverses Apo2L resistance of HTLV-1-infected T-cell lines. Because NF-κB could play a role in the regulation of survival of HTLV-1-infected T-cell lines and primary ATL cells (43) and NF-κB can modulate Apo2L-induced cell death (6, 9, 21, 55), we investigated whether inhibition of NF-κB transcriptional activity could modulate the response of HTLV-1-infected T-cell lines to Apo2L. The proteasome inhibitor \( N\)-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (LLnL) (21), which is known to inhibit the activation of NF-κB by blocking the degradation of the IκB protein, had a toxicity profile in HTLV-1-infected T-cell lines but not in uninfected T-cell lines. Therefore, HTLV-1-infected T-cell lines were pretreated with a low toxic concentration (25 \( \mu \)M in C5/MJ and 1 \( \mu \)M in MT-2 and SLB-1 cells) of LLnL, followed by 24-h exposure to Apo2L, and then WST-8 was added for the last 4 h of incubation. As shown in Fig. 7A, LLnL treatment inhibited NF-κB DNA binding but not AP-1 DNA binding. LLnL reversed the Apo2L resistance of C5/MJ, SLB-1, and MT-2 cells.
FIG. 6. Expression of Apo2L receptors, FLIP mRNA, and active Akt in T-cell lines. (A) RT-PCR analysis of human T-cell lines for expression of Apo2L receptors (DR4, DR5, DcR1, and DcR2) and FLIP. Total RNA was prepared from the indicated T-cell lines. β-Actin served as an internal control in the RT-PCR procedure. NB4 cells were used as a positive control for DR4, DR5, DcR1, and DcR2. (B) Active Akt in T-cell lines. Western blot analyses were performed with anti-phospho-Akt, anti-Akt, or anti-actin antibody. A schematic of the four Apo2L receptors is shown at the top. (C) Cell surface expression of Apo2L receptors on T-cell lines. T-cell lines were stained with control mouse immunoglobulin G1 or anti-human DR4, DR5, DcR1, and DcR2 monoclonal antibodies and analyzed by flow cytometry. Shaded and unshaded peaks correspond to specific and control stainings, respectively.
FIG. 7. Inhibition of NF-κB reverses Apo2L resistance of HTLV-1-infected T cells. (A) EMSA of NF-κB and AP-1 activation status in C5/MJ cells before and after exposure to the NF-κB inhibitor LLnL at 25 μM. Constitutive activation of NF-κB, but not AP-1, was reduced in the presence of LLnL.

(B) WST-8 assays indicate that pretreatment with LLnL overcomes the Apo2L resistance of HTLV-1-infected T-cell lines. Cells were either not treated or treated with LLnL (25 μM in C5/MJ and 1 μM in the residual cell lines) for 2 h prior to the addition of Apo2L (100 ng/ml). After a further 24 h, cell survival was assessed by WST-8 assays. The data are the mean plus standard deviation of three independent experiments.

(C) Expression of DR4 and DR5 increases following LLnL treatment (+). (D) Expression of intracellular apoptosis regulator proteins in C5/MJ cells treated with LLnL. Immunoblot analysis in C5/MJ cells treated with (+) and without (−) LLnL for 24 h. Levels of actin are shown for confirmation of equal protein loading.
(Fig. 7B). We next sought to identify the molecular target of LLnL in the apoptotic machinery. C5/MJ cells were treated for 24 h with LLnL, and surface expression levels of DR4, DR5, DcR1, and DcR2 and protein levels of IκBα, Bcl-2, Bcl-xL, Bax, LAP-2, and survivin were then examined by flow cytometry and immunoblotting, respectively. As shown in Fig. 7D, the increased response to Apo2L could not be attributed to changes in levels of proapoptotic and antiapoptotic proteins, although LLnL inhibited the degradation of IκBα. On the other hand, among the Apo2L receptors, surface expression levels of DR4 and DR5 were significantly increased after treatment with LLnL (Fig. 7C). A similar experiment was also performed in C5/MJ cells using another NF-κB-specific inhibitor, Bay 11–7082, which blocks the phosphorylation of IκBα (51). Bay 11–7082 also enhanced the surface expression of DR4 and DR5 on C5/MJ cells (data not shown). Considered together, these results suggest that modulation of Apo2L sensitivity by NF-κB inhibitors is probably due to changes in the expression levels of DR4 and DR5 receptors.

**DISCUSSION**

Apo2L is a member of the superfamily of TNF-related ligands that potently induces apoptosis of a wide range of cancer cells while sparing normal cells (3, 10, 14, 16, 17, 28, 34, 61, 64, 72). We have shown for the first time that HTLV-1-infected T-cell lines and primary ATL cells are resistant to Apo2L-induced apoptosis. Resistance to Apo2L could not be attributed to expression levels of Apo2L receptors, FLIP, or active Akt. We showed that high NF-κB activity inversely correlated with Apo2L sensitivity. Interestingly, we showed that LLnL, a proteasome inhibitor, could overcome resistance in Apo2L-resistant HTLV-1-infected T cells. In the present study, we attempted to identify the molecular mediator of LLnL on apoptosis. The sensitization of resistant cells to Apo2L by LLnL could not be attributed solely to changes in levels of proapoptotic and antiapoptotic proteins. We found that sensitization to Apo2L by LLnL was associated with up-regulation of DR4 and DR5, which occurred in cells treated with Bay 11–7082, an inhibitor of the phosphorylation of IκBα (Fig. 7C and data not shown). The expression levels of DR4 and DR5 on LLnL- or Bay 11–7082-treated cells were much higher than those on Apo2L-sensitive cell lines. Although DR4 and DR5 expression levels were not regulated by NF-κB, inhibition of NF-κB activation might augment DR4 and DR5 expression and interfere with unknown protective proteins in the apoptotic signal cascade.

Our findings are consistent with the hypothesis that active infection with HTLV-1 leads to disturbance of apoptosis regulation and constitutive expression of inducers of apoptosis. The major findings of the present study were that (i) Apo2L is expressed in HTLV-1-infected T-cell lines, as well as primary ATL cells; (ii) Tax is responsible for the expression of Apo2L through the NF-κB pathway; and (iii) an NF-κB binding site in the Apo2L promoter between −74 and −65 is required for Tax-induced Apo2L activity. Experiments using Tax mutants indicated that the NF-κB pathway is required for the full activation of the Apo2L promoter by Tax. Furthermore, blocking NF-κB activation by truncated forms of IκBα and IκBβ clearly inhibited Apo2L activation by Tax. These findings suggest that NF-κB activation may be a prerequisite for increased T-cell Apo2L activation in response to Tax. In this study, we identified the signaling components NIK and IKKs as likely participants in Tax-mediated Apo2L activation. Activation of the NF-κB pathway by Tax has been extensively investigated. In support of our findings, Tax expression has been shown to promote phosphorylation and activation of IKKα and IKKβ by increasing the activity of the upstream kinase NIK (11).

Although Apo2L mRNA expression is detected in various cells and tissues, including PBLs (64), regulation of its expression remains largely unknown. A recent study demonstrated that the induced expression of Apo2L in Jurkat cells following treatment with a variety of stimuli, such as phorbol myristate acetate, is linked to two NF-κB binding sites, κB2 (located between −384 and −375) and κB1 (located between −264 and −255), within the Apo2L promoter (4). However, deletion of these two sites did not diminish promoter activation by Tax. Among the multiple regulatory domains identified in the Apo2L upstream promoter, another NF-κB binding site, located at −74 to −65 upstream from the transcription initiation site, was studied. The Tax-responsive element within the 5′ regulatory sequences of the Apo2L gene was localized in this site. This NF-κB site was able to bind to p50, p65, and c-Rel in a Tax-dependent manner. Deletion of this site resulted in loss of Tax responsiveness.

Previous studies reported that Apo2L is responsible for the activation-induced death of T cells during human immunodeficiency virus infection (24, 25). Related studies also demonstrated that reovirus-, measles virus-, and human cytomegalovirus-infected cells are rendered cytotoxic via the Apo2L pathway (8, 56, 62), indicating that virus-infected cells express enhanced levels of Apo2L, which is responsible for virus-induced apoptosis (8, 24, 25, 56, 62). Accordingly, it is interesting that primary ATL cells, which phenotypically resemble activated T cells, express up-regulated Apo2L and escape Apo2L-mediated elimination.

Tax is unambiguously the cellular transforming growth factor for HTLV-1. Activation of NF-κB by Tax has been proposed as the causal viral mechanism for ATL. Accordingly, although it has yet to be contemplated, ATL therapy, in principle, would benefit from interruption of NF-κB activation (43). In summary, high NF-κB activity confers resistance to Apo2L and Apo2L expression on HTLV-1-infected T-cells. Inhibitors of NF-κB may be useful clinically as adjunctive agents in the treatment of ATL.

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RETRACTION

Resistance to Apo2 Ligand (Apo2L)/Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)-Mediated Apoptosis and Constitutive Expression of Apo2L/TRAIL in Human T-Cell Leukemia Virus Type 1-Infected T-Cell Lines

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