NEOPLASIA

Constitutive Activation of NF-κB in Primary Adult T-Cell Leukemia Cells

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Human T-cell leukemia virus type I (HTLV-I) is an etiologic agent of adult T-cell leukemia (ATL). The viral protein Tax induces the activation and nuclear translocalization of transcription factor NF-κB, which is proposed to play a crucial role in the transformation of T cells by HTLV-I. However, the HTLV-I genes including Tax are not expressed significantly in primary leukemic cells from ATL patients. In this study, we examined the basis for NF-κB activation in freshly isolated leukemic cells from ATL patients. We found that leukemic cells from ATL patients, like HTLV-I-infected T-cell lines, display constitutive NF-κB DNA binding activity and increased degradation of IκBα (an inhibitor of NF-κB). Whereas the NF-κB binding activity in Tax-expressing T-cell lines consisted mostly of p50/κB, fresh ATL samples contained p50 and p50/p65 heterodimers. One T-cell line derived from ATL leukemic cells, TL-Om1, displayed constitutive NF-κB activity, as well as enhanced degradation of IκBα, despite the lack of detectable Tax expression. Interestingly, the NF-κB in TL-Om1 consists of p50 and p50/p65 like that in fresh primary leukemic cells. Our results suggest that activation of NF-κB occurs through a Tax-independent mechanism in leukemic cells of ATL patients, possibly due to differential NF-κB subunit activation.

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MATERIALS AND METHODS

Cells. MT-2, HUT-102, SLB-1, C5/MJ, and TL-Om1 are human T-cell lines infected with HTLV-I. Jurkat, MOLT-4, and H-9 are human T-cell lines. These T-cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, and antibiotics.

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were used, and the sequences are 5'-gatcCGGCAGCGATCCCTCCTC-3', underlined sequences represent the kB binding sequence of IL-2Rα gene was 5'-gatcCGGCAGCGATCCCTCCTC-3', containing the consensus sequence of the octamer binding motif (underlined) was used to identify specific binding of the transcription factor Oct-1. This transcription factor regulates transcription of a number of so-called housekeeping genes. For competition studies, oligonucleotides containing mutant kB and two kB motifs derived from the human immunodeficiency virus type 1 long terminal repeat (LTR) were used, and the sequences are 5'-gatcCGGCAGACACCTTCCTCCTC-3'. For the preparation of a probe in electrophoretic mobility shift assay (EMSA), a radiolabeled double-stranded oligonucleotide was prepared by annealing and filling in the overhang with the Klenow fragment of DNA polymerase I in the presence of [α-32P]deoxyadenosine triphosphate (dATP).

Preparation of nuclear extracts. Nuclear extracts were prepared as described by Antalis et al.27 with modifications. Cells (10⁷) were washed twice with cold PBS and the cell pellet was suspended in 400 µL of hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], 2 mM aminoethyl-benzensulfonfluoride [AEBSF], and 0.2% Nonidet P-40) for 10 minutes at 4°C. Nuclei were prepared by microcentrifugation for 5 minutes at 4°C. The nuclear pellet was suspended in 75 µL of buffer C (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 2 mM AEBSF, 33 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL E-64, and 10 µg/mL pepstatin A) and incubated for 30 minutes at 4°C with brief mixing. The mixture was microcentrifuged (15,000 cpm) for 15 minutes at 4°C. Protein concentration was measured by using the Bradford assay (Bio-Rad, Richmond, CA).

EMSA. As previously described, nuclear extracts (5 µg of protein) were preincubated in a 20-µL total reaction volume containing 10 mM HEPES, 0.5 mM HEPES, 1 mM EDTA, 1 mM DTT, 5 µL, and 1 µg of polydeoxyinosinic-deoxycytidylic acid (Pharmacia, Piscataway, NJ) for 15 minutes at room temperature. The reaction mixture was then incubated with the radiolabeled oligonucleotide (50,000 cpm) for 15 minutes at room temperature. The samples were analyzed by electrophoresis in a 4% nondenaturing polyacrylamide gel with 0.25 × TBE buffer (22.3 mM Tris, 22.2 mM boric acid, and 0.5 mM EDTA). The gels were dried and analyzed by autoradiography.

Plasmids and transfection. A reporter plasmid kB-LUC (a kind gift from Dr J. Fujisawa, Kansai Medical University, Osaka, Japan) was constructed by inserting five repeats of the NF-κB binding sequence of the IL-2Rα gene fused to an enhancerless promoter of HTLV-I into pGGL2-Basic containing the luciferase gene, nN-LUC.21 The NF-κB expression plasmids, human p50, p65, and c-Rel, were the generous gifts of Dr K. Yamamoto (Kanazawa University, Kanazawa, Japan). Transfections were performed by electroporation.8 In all cases, the reference plasmid pRL-CMV, which contains the Renilla luciferase gene under the control of the cytomegalovirus immediate early enhancer/promoter, was co-transfected to correct for transfection efficiency. For luciferase assay, the transfected cells were lysed in a lysis reagent (Toyo Ink Co, Tokyo, Japan), and luciferase activities were measured according to the manufacturer's procedures. Each assay was independently repeated at least three times.

RESULTS

HTLV-I–infected T-cell lines constitutively express a factor mediating activation of NF-κB element. As an initial test of the activity of NF-κB in HTLV-I–infected T-cell lines, a transfection analysis was performed using a reporter construct, kB-LUC, containing a luciferase gene under the control of the NF-κB binding site from the IL-2Rα gene. The level of luciferase activity detected in uninfected cells transfected with kB-LUC was similar to that of cells transfected with nN-LUC (Fig 1A). On the other hand, in all of the five HTLV-I–infected cell lines, the kB-LUC reporter gene was 9 to 13 times more active than nN-LUC, indicating enhanced NF-κB activity in HTLV-I–infected cell lines.
maintain NF-κB–dependent transcription in HTLV-I–infected T-cell lines. Potential alterations in cellular gene activation associated with the Tax-independent activation of NF-κB in TL-Om1 were next studied by measuring expression of the IL-2Rα gene, possibly due to the activation of NF-κB.

**Lower protein levels of IkBα in HTLV-I–infected T-cell lines.** In a variety of cell lines, NF-κB is localized in the cytoplasm through binding to specific cytoplasmic molecules called IkBα. After cellular stimulation by multiple inducers, activation of signal transduction cascades leads to the degradation of IkBα. In agreement with prior studies, the protein level of IkBα was significantly lower in HTLV-I–infected cell lines (Fig 3A). More importantly, TL-Om1 cells, which do not express Tax, have reduced IkBα protein at a similar level to HTLV-I–infected cell lines expressing Tax. These results suggest that IkBα protein expression is downregulated in TL-Om1 by a Tax-independent mechanism.

**Fig 1.** (A) HTLV-I–infected T-cell lines express a constitutive NF-κB–related activity. Various cell lines were transfected with 10 µg of a reporter plasmid containing the luciferase gene fused to five repeats of the κB motif of the IL-2Rα gene and enhancerless promoter of HTLV-I (κB-LUC). After 24 hours of incubation, lysates were prepared and assayed for luciferase activity. Ratio of luciferase activity in extracts of κB-LUC–transfected cells is expressed to that for dN-LUC–transfected cells. The results represent the mean of three experiments. (B) Northern blot analysis of Tax in various cell lines. Total RNA (20 µg of each) was isolated from various cell lines. Jurkat, MOLT-4, and H-9 are HTLV-I–uninfected T-cell lines. MT-2, HUT-102, SLB-1, TL-Om1, and CS/MJ are HTLV-I–infected T-cell lines. Blots were sequentially hybridized, exposed, stripped, and rehybridized with Tax and GAPDH probes.

**Fig 2.** Expression of IL-2Rα at protein (A) and mRNA (B) levels in various cell lines. (A) The percentages of positive cells for IL-2Rα are shown. (B) Total RNA (20 µg of each) was isolated from various cell lines and assessed for IL-2Rα or GAPDH mRNA expression.
Overexpression of IκBa mRNA in HTLV-I–infected T-cell lines.

Next, we performed Northern blot analysis of IκBa mRNA in the T-cell lines characterized above. As shown in Fig 3B, the expression of IκBa mRNA in TL-Om1, as well as Tax-expressing cell lines, was strikingly higher than in uninfected ones. Thus, decreased IκBa protein level detected in HTLV-I–infected cell lines, including TL-Om1, is not due to impaired IκBa gene activity, but due to the rapid turnover of IκBa. It has been reported previously that Tax disrupts feedback regulation of NF-κB through destabilization of IκBa, thus establishing constitutive activation of NF-κB. JPX-9 cells are derived from a human T-cell line Jurkat, and they are permanently transfected with a metallothionein promoter-driven Tax expression vector. As a result of the expression of Tax in JPX-9 cells by CdCl2 treatment, IκBa mRNA expression was markedly upregulated (Fig 4A). However, the total protein levels for IκBa remained constant (Fig 4B), possibly indicative of a more rapid turnover of IκBa. Taken together, these results show that Tax-mediated destabilization of IκBa is the mechanism for reduction of IκBa protein in HTLV-I–infected cell lines expressing Tax. These data also suggest that rapid degradation of IκBa is maintained by mechanisms other than Tax in TL-Om1 cells.

Enhanced IκBa degradation in primary ATL cells. We had postulated that the IκBa/NF-κB pathways may already be activated in ATL leukemic cells in vivo. Therefore, we examined whether the expression of IκBa in freshly obtained PBMC of ATL patients is upregulated. PBMC were isolated from peripheral blood of seven ATL patients and RNA was prepared for Northern blot analysis. As shown in Fig 5A, IκBa mRNA levels

![Diagram](https://example.com/diagram.png)
levels in freshly isolated PBMC of ATL patients were much higher than those in PBMC from healthy control subjects. Furthermore, IL-2Rα expression was stronger in primary ATL cells than in normal PBMC (Fig 5A). These results are similar to the high expression of IκBα and IL-2Rα mRNA observed in HTLV-I–infected T-cell lines. In addition, Tax expression in the primary cells isolated from these patients was hard to estimate quantitatively with Northern and Western blot analyses (data not shown).

We next determined the protein levels of IκBα in blood mononuclear cells from patients with ATL and from healthy subjects. Whole-cell extracts were prepared from PBMC and subjected to Western blot analysis (Fig 5B). The IκBα reactivity band was strongly visible in PBMC from healthy subjects. In contrast, IκBα reactivity was weak in PBMC derived from two cases (ATL patients 5 and 6). The remaining five samples did not show apparent reduction of the bands. However, these results were not influenced by the contents of leukemic cells in these samples. While IκBα mRNA levels were dramatically upregulated in ATL cells, the total protein levels did not increase, possibly indicative of protein degradation.

Constitutive NF-κB binding activity in HTLV-I–infected T-cell lines and primary ATL cells. To determine whether degradation of IκBα protein in HTLV-I–infected T-cell lines was associated with NF-κB activation, nuclear extracts of cells were subjected to EMSA. In uninfected T-cell lines, no NF-κB–specific protein DNA complexes were detected in nuclear extracts by EMSA (Fig 6A, lanes 1 through 3). In contrast, enhanced NF-κB activity was present in each of the HTLV-I–infected T-cell lines tested (Fig 6A, lanes 4 through 8). The specificity of the binding activities was shown by competition with excess wild-type and mutant oligonucleotides (data not shown). Constitutive NF-κB binding activity in MT-2 cells was composed predominantly of c-Rel and p50, as antibodies against c-Rel and p50 partially inhibited formation of the NF-κB DNA complex and also produced a shifted complex (Fig 6B, left panel). Similar analyses showed that c-Rel and p50 subunits represented the main DNA binding components in HUT-102, SLB-1, and C5/MJ cells (data not shown). In TL-Om1 cells, two forms of complexes were detected (Fig 6A). Incubation of the nuclear extracts with various antibodies represented that the upper band contains p50 and p65, and the lower band contains only the p50 (Fig 6B, right panel).

We determined whether degradation of IκBα protein in primary ATL cells involved abnormal NF-κB–specific DNA binding activity. To address this question, nuclear extracts of PBMC obtained from seven patients with ATL and healthy subjects, the same samples as analyzed in Fig 5, were subjected to EMSA (Fig 6A). Nuclear extracts were prepared from PBMC and subjected to Western blot analysis (Fig 5B). The IκBα reactivity band was strongly visible in PBMC from healthy subjects. In contrast, IκBα reactivity was weak in PBMC derived from two cases (ATL patients 5 and 6). The remaining five samples did not show apparent reduction of the bands. However, these results were not influenced by the contents of leukemic cells in
to EMSA. In extracts from healthy subjects, NF-κB–specific complex was detected (Fig 7A, left panel). This protein DNA complex was composed primarily of p50; interaction with anti-p65, c-Rel, and p52 antibodies was negligible (Fig 7D, right panel). On the other hand, two forms of complexes were detected, an upper band and a lower band in extracts from primary ATL cells (Fig 7A, left panel). Although the observed complexes formed broad bands in patients 2, 5, 6, and 7, lighter exposure of the gel showed two bands in these cases (Fig 7A, right panel). The specificity of these complexes was verified by competition with an excess of wild-type and mutant oligonucleotides (Fig 7C). Incubation of the protein DNA complex with anti-p50 resulted in almost complete supershift of the upper and lower migrating bands (Fig 7D, left panel). Incubation with anti-p65 reduced only the upper band completely. Further, anti-c-Rel and p52 antibodies failed to shift any bands (Fig 7D, left panel). This result suggests that the upper band contains p50 and p65, and the lower band contains the p50 homodimers. No differences between ATL patients and healthy subjects in binding to the octamer motif on DNA were found (Fig 7B).

**Transactivational activities of different members of the NF-κB/Rel family at the IL-2Rα κB site.** To determine the transcription activity of different subunit members of the NF-κB/Rel family, expression plasmids for three κB-related proteins, p50, p65, and c-Rel, were cotransfected in Jurkat cells with a reporter plasmid, κB-LUC. As shown in Fig 8, expression of p65 and c-Rel, by themselves or in combination with p50, led to an increase in luciferase activity, while expression of p50 was devoid of effect on the reporter gene transcription. These results recapitulate the observed EMSA and IL-2Rα expression data, indicating that p65 or c-Rel binding can activate NF-κB–dependent transcription. However, p50 homodimers can bind to the κB element in the IL-2Rα, but are unable to support transcriptional activation.

**DISCUSSION**

We analyzed for the first time the functional status of the NF-κB/IκBα pathway in primary leukemic cells from ATL patients. In all patients, NF-κB protein’s DNA binding activity was detected by EMSA in the leukemic cell extracts. Furthermore, this study shows that in contrast to healthy subjects, IκBα in freshly isolated leukemic cells from patients with ATL has...
already undergone proteolytic degradation. IkBα mRNA was upregulated in ATL cells of patients. However, IkBα protein levels did not increase in concert with the highly elevated expression of the mRNA. Furthermore, IkBα protein levels were significantly lower in two samples. Thus, increased turnover of the IkBα protein in leukemic cell extracts of patients with ATL was associated with activation of NF-κB. These results were similar to those observed in infected and Tax-expressing T-cell lines. Recently, like IkBα, Tax-mediated breakdown of IkBβ was reported. In preliminary experiments, little or no detectable amount of IkBβ was found in primary ATL cells. Together, these results suggest that the degradation of IkBα and IkBβ may play a role in the constitutive activation of NF-κB in ATL.

Importantly, the identity of the shifted bands was confirmed by supershifting with NF-κB-specific antibodies. We observed that the dynamic alterations in NF-κB binding activity occurred in ATL patients. In all patients’ extracts, the DNA protein complex consists of p50/p65 heterodimers and p50 homodimers. In contrast, NF-κB binding activity in healthy subjects was composed of p50 homodimers alone. Interestingly, cotransfection with various NF-κB subunits indicated that p65 and c-Rel, but not p50, was able to activate transcription at the IL-2Rα kxB site. The observation that constitutive binding of heterodimeric p50/p65 correlated with expression of IL-2Rα in ATL patients supports the notion that in vivo p50/p65 heterodimers and p50 homodimers can bind to the kxB element in the IL-2Rα promoter, but only p50/p65 heterodimers can activate transcription.

HTLV-I Tax has been shown to induce a degradation of IkBα, which may contribute to the nuclear expression of a number of NF-κB/Rel species. NF-κB is known to mediate the Tax-dependent transactivation of IL-2Rα, IL-1α, IL-6, IL-8, and tumor necrosis factor β genes. Considering the previously reported enhanced production of these inflammatory cytokines and the increased expression of surface molecules such as IL-2Rα in ATL patients, it is not surprising that activated NF-κB/IkBα pathway was seen in ATL cells. However, consistent with previous studies, primary leukemic cells isolated from ATL patients did not express tax at a significant level. Because viral expression in vivo differs significantly from that observed in cell lines in vitro, it is of interest to understand the differences of NF-κB activation between leukemic cells in vivo and cell lines established in vitro. Tax induces predominantly the c-Rel-containing complexes and transcriptionally activates the c-Rel gene. Consistent with these studies, in HTLV-I–infected T cells that constitutively express high levels of Tax, p50 and c-Rel were the major DNA binding components; similar c-Rel-containing complexes were also observed previously, suggesting Tax-independent mechanisms by which primary ATL cells exhibit persistent nuclear expression of p50/p65 heterodimers.

In this study, we have shown that the ATL-derived TL-Om1 cell line fails to express detectable levels of Tax. However, TL-Om1 was shown to carry full-length proviral genome of HTLV-I by Southern blot analysis. It was also found that the HTLV-I LTR function was not enhanced in TL-Om1, as the LTR luciferase activity in TL-Om1 was one fourteenth of that in MT-2. Furthermore, Tax induced transcription from the LTR in TL-Om1 (data not shown). We have also shown the rapid degradation of IkBα in TL-Om1 cells, which may be directly involved in constitutive NF-κB activation. This line still expresses IL-2Rα mRNA and protein at high levels. In addition, TL-Om1 cells express chemokine genes such as IP-10 and I309, and intercellular adhesion molecule 1 at the same levels as those in Tax-expressing T-cell lines infected with HTLV-I. These results suggest that Tax is not the only mechanism for constitutive expression of certain cellular genes in HTLV-I–infected T cells. We believe in the possibility of Tax-independent mechanisms operating for constitutive NF-κB activation in leukemic cells of ATL patients. TL-Om1 should help to identify new targets of NF-κB by comparing gene expression between this cell line and other Tax-expressing cell lines.

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