Activation of AID by human T-cell leukemia virus Tax oncoprotein and the possible role of its constitutive expression in ATL genesis

Chie Ishikawa1,2, Sawako Nakachi1,3, Masachika Senba4, Manabu Sugai5 and Naoki Mori1,6,*

1Department of Microbiology and Oncology, Graduate School of Medicine, University of the Ryukyus, Nishihara, Okinawa 903-0215, Japan, 2Transdisciplinary Research Organization for Subtropics and Island Studies, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan, 3Department of Endocrinology, Metabolism and Hematology, Graduate School of Medicine, University of the Ryukyus, Nishihara, Okinawa 903-0215, Japan, 4Department of Pathology, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan and 5Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto 606-8507, Japan

*Present address: Department of Internal Medicine, Otoromachi Medical Center, 1-3-1 Uenoya, Naha, Okinawa, Japan

To whom correspondence should be addressed. Department of Internal Medicine, Otoromachi Medical Center, 1-3-1 Uenoya, Naha, Okinawa 900-0011, Japan. Tel: +81 98 867 2116; Fax: +81 98 861 2398; Email: naokimori50@gmail.com

Adult T-cell leukemia (ATL) is a T-cell malignancy associated with human T-cell leukemia virus type 1 (HTLV-1). Mutations of tumor suppressor genes have been described in ATL. Although Tax, a product of HTLV-1, is associated with cellular genetic aberrations, the mechanisms of such association are not fully clear. Activation-induced cytidine deaminase (AID) is involved in somatic DNA alterations of the immunoglobulin gene for amplification of immune diversity. However, inappropriate expression of AID acts as a genomic mutator that contributes to tumorigenesis. To gain insight into the molecular mechanism underlying the emergence of somatic mutations in various genes during leukemogenesis, we examined the expression of AID. HTLV-1-infected T-cell lines and ATL cells expressed high levels of AID compared with uninfected T-cell lines and normal peripheral blood mononuclear cells (PBMCs). Immunohistochemistry showed AID-positive ATL cells in lymph nodes and skin lesions. Infection of a human T-cell line and normal PBMCs with HTLV-1 induced AID expression. Tax transcriptionally activated AID gene through both the nuclear factor-kappaB subunit p50 and cyclic adenosine 3',5'-monophosphate response element-binding protein signaling pathways. p50, which lacks a transactivation domain, interacted with the transcriptional activator Bcl-3 in HTLV-1-infected T cells. Thus, activation of p50/Bcl-3 complexes in T cells in response to Tax might explain the constitutive expression of AID in HTLV-1-infected T cells. The constitutive expression of AID in ATL cells can be speculated to result from mutations induced by the Tax-activated AID and/or other Tax-associated mutagenic mechanisms during the pre-leukemic stage, which cause functional modification within the AID promoter or in any of its cellular regulatory activator proteins.

Introduction

Adult T-cell leukemia (ATL) is a highly aggressive malignancy of mature CD4+ T cells etiologically caused by human T-cell leukemia virus type 1 (HTLV-1) (1). ATL develops after a long period of latency, usually 40–60 years. The vast majority of infected persons remain clinically asymptomatic, whereas only 2–5% develop neoplasia. After infection of the T cells, ATL is thought to develop after a multitude of events including both genetic and epigenetic changes in the cell over time (2). Despite intensive studies on HTLV-1, the pathogenetic mechanism(s) involved leukemogenesis remain not fully understood. However, the viral Tax protein is widely regarded as a key factor in this mechanism because of its capacity to stimulate or repress the synthesis or function of many regulatory factors involved in a wide range of normal and pathogenic cellular processes (3). By constitutive induction of regulatory factors involved in activation of T-cell replication, Tax protein can set infected T cells into a continuous uncontrolled replication. Tax affects both the fidelity of chromosomal segregation (aneuploidiogenetic) (4) and the mismatch repair (clastogenic) (5) functions. Mechanistically, Tax has been shown to abrogate the mitotic checkpoint function (3) and lead to miscounted chromosomes in HTLV-1-transformed T cells (6). Tax has also been shown to interfere with most DNA repair mechanisms, thus further intensifying the genome instability of these cells (7). Usually, cells that suffer from mitotic checkpoint dysfunction, or cannot repair damage imposed on their DNA, enter into cell-cycle arrest or apoptosis. In contrast, HTLV-1-infected T cells are protected by Tax from both these responses (7). Based on these pleiotropic activities, Tax acts as a potent oncoprotein capable of transforming cultured animal cells, inducing tumors in transgenic mice and immortalizing or transforming human primary T cells (7). Thus, the oncogenic potency of Tax is thought to initiate the leukemic process leading to ATL (7).

It is widely recognized that mutations of oncogenes, tumor suppressor genes and genomic stability genes play pivotal roles in carcinogenesis (8). Despite remarkable progress in our knowledge of the molecular mechanisms of individual cancer-related genes, surprisingly little is known about the fundamental aspects of when and how mutations are introduced into what kind of cell populations.

To address this problem, a new mechanism of mutagenesis in cancer development has been proposed recently (9,10). It is hypothesized that at least certain cancer-related mutations are introduced by activation-induced cytidine deaminase (AID), an enzyme expressed in activated B lymphocytes, and is required for somatic hypermutation and class-switch recombination of antibody genes (11). The hypothesis is based on the following observations: (i) AID can induce mutations in non-B cells (12), (ii) AID transgenic mice develop various tumors, including T-cell lymphoma and lung microadenoma (13) and (iii) AID can be induced in human hepatic and gastric epithelial cells when challenged with pathogens such as hepatitis C virus and Helicobacter pylori (14,15). On the basis of this evidence, AID is a potential candidate mutagen in human cancers.

Based on the above background, we hypothesized that aberrant expression of AID in HTLV-1-infected T cells plays a role in the enhanced genetic susceptibility to mutagenesis during leukemogenesis. In the present study, we examined the expression of AID in various T-cell lines and clinical samples from patients with ATL. The purpose of the study was to determine whether Tax induces the expression of AID and the molecular mechanism by which AID is induced by Tax. Our findings may provide a molecular explanation for the clastogenic effect of Tax.

Materials and methods

Cells

HTLV-1-infected T-cell lines MT-2 (16), MT-4 (17), CEM/18, TL-Omrl (19) and ED-40515(-) (20), HTLV-1-uninfected T-cell lines Jurkat, MOLT-4 and CCRF-CEM and Burkitt lymphoma cell line Ramos were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum.
AID expression in ATL

(FBS; Biological Industries, Kibbutz Beitz Haemek, Israel), 50 U/ml of penicillin and 50 µg/ml of streptomycin. MT-2, MT-4 and C5/14 are HTLV-1-transformed T-cell lines and constitutively express viral genes including Tax. TL-On1 and ED-40515(-) are T-cell lines of leukemic cell origin that were established from patients with ATL and do not express viral genes. JFX-9 is a subline of JFX that expresses Tax under the control of the metallothionein gene promoter (21). Peripheral blood mononuclear cells (PBMCs) were isolated from 6 healthy volunteers, 20 patients with acute-type ATL, 1 patient with B-lineage acute lymphoblastic leukemia (B-ALL) and 2 patients with T-lineage acute lymphoblastic leukemia (T-ALL) using Ficoll–Paque density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden). Bone marrow samples from five patients with B-ALL were also obtained. For the usage of activated T cells, PBMCs were stimulated with 10 µg/ml phytohemagglutinin for 72 h. All samples were collected at the time of admission to hospital before the patients started chemotherapy. The diagnosis of ATL was based on clinical features, hematologic findings and the presence of anti-HTLV-1 antibodies in the sera. Monoclonal HTLV-1 provirus integration into the DNA of leukemic cells was confirmed by Southern blot hybridization in all patients (data not shown). This study was approved by the Institutional Review Board of the University of the Ryukyus. Informed consent was obtained from all blood and tissue donors in accordance with the Declaration of Helsinki.

Reagents

N-acetyl-seryl-leucyl-tyrosyl-leucyl-norleucinal and Bay 11-7082 were purchased from Sigma–Aldrich (St Louis, MO) and Calbiochem (La Jolla, CA), respectively. Human recombinant p50 was obtained from Promega (Madison, WI).

Reverse transcription–polymerase chain reaction

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. First-strand complementary DNA was synthesized from 1 µg total cellular RNA using a RNA-polymerase chain reaction (PCR) kit (Takara Bio, Otsu, Japan) with random primers. The sequences of the primers were described previously (14,22–26). The p65 primer pairs were as follows: p65 sense, 5’-GGGCGCAAGACCTTAGTCTGCGGTAGTAAAC-3’ and antisense, 5’-GGGCGCCATAGAACAACTGACC-3’; the setting for semi-quantitative reverse transcription (RT)–PCR for each gene was as follows: 25 cycles for p50/p100; 30 cycles for p65, Bcl-3 and Tax; 35 cycles for AID and apolipoprotein B messenger RNA-editing enzyme, catalytic polypeptide-like 3 (APOBEC3)G and 28 cycles for β-actin. The PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining. The obtained bands of amplified DNA were quantified using AlphaEase FC software (Alpha Innotech Corporation, San Leando, CA). Data were normalized to β-actin loading controls.

Western blot analysis

Cells were lysed and equal amounts of protein (20 µg) were subjected to electrophoresis on sodium dodecyl sulfate–polyacrylamide gels, followed by transfer to polyvinylidene difluoride membrane and sequential probing with a rabbit monoclonal antibody to AID (Cell Signaling Technology, Beverly, MA), rabbit polyclonal antibody to Bcl-3 (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal antibodies to actin (NeoMarkers, Fremont, CA) and Tax, Lit-4 (27). The bands were visualized with an enhanced chemiluminescence kit (Amersham Biosciences, Pisicatway, NJ).

Immunohistochemical analysis

Biopsy samples were taken from the lesional skin of 10 patients with ATL and lymph nodes of 6 patients with ATL. AID immunohistochemistry was performed using a mouse anti-AID monoclonal antibody (Zymed Laboratories, South San Francisco, CA) after pretreatment of the deparaffinized tissue sections with ready-to-use proteinase K (Dako, Carpinteria, CA). The sections were counterstained with methyl green, hydrated in ethanol and cleaned in xylene and mounted. The stained cells were examined under a light microscope (Axioskop 2 plus; Zeiss, Jena, Germany) with an Achroplan x40/0.65 lens (Zeiss). Images were acquired with an AxioCam MRC camera and AxioVision 3.1 software (Zeiss).

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 20 min at 37°C. Fixed cells were washed with phosphate-buffered saline (PBS) containing 7% of PBS twice and permeabilized with PBS containing 0.1% Triton X-100 for 10 min at room temperature. The cells were washed with PBS containing 7% of PBS once and resuspended in PBS/7% PBS containing rabbit polyclonal antibody to Bcl-3 (Santa Cruz Biotechnology) and mouse monoclonal antibody to p50 (Santa Cruz Biotechnology) for 20 min at room temperature. The cells were washed with PBS/7% PBS twice and resuspended in PBS/7% PBS containing Alexa Fluor 488-labeled goat anti-mouse immunoglobulin G (IgG) and Alexa Fluor 546-labeled goat anti-rabbit IgG (Invitrogen) for 20 min at room temperature. The nuclei were stained with Hoechst 33342 (Wako Pure Chemical Industries, Osaka, Japan). Finally, the cells were washed with PBS twice and observed under a Leica DMi6000 microscope (Leica Microsystems, Wetzlar, Germany). Mounted coverslips were imaged through a ×63 oil immersion lens (NA1.4) on a Leica TCS SP5 confocal system.

HTLV-I infection by cocultivation

HTLV-1-infected MT-2 cells were pretreated with 200 µg/ml of mitomycin C (MMC) for 60 min at 37°C, pipetted vigorously and washed three times with PBS. PBMCs from healthy donors (5 × 10^6 per well) and MMC-treated MT-2 cells (5 × 10^4 per well) were cocultured in a 24-well plate in the presence of 10 ng/ml of interleukin (IL)-2. The culture medium was changed, with fresh medium supplemented with IL-2 every 3 days. T8Y-3/MT-2 was established from the IL-2-dependent human T-cell line T8Y-3 cocultured with MMC-treated MT-2 cells and was capable of growth completely independent of IL-2 (28).

Transfection and luciferase assay

Various expression vectors for Tax (pMWT-2 Tax) and its mutants (Tax M22 and Tax M30) were described previously (29). Tax M22 has an amino acid substitution at codons 130 and 131 from Thr–Leu to Ser–Ala. Tax M30 has a frameshift mutation at codon 134. Tax M22 does not activate transcription factors in a nuclear adenosine 3',5'-monophosphate response element (CRE), which mediates the Tax-dependent activation of the HTLV-1 long terminal repeat but not the nuclear factor-kappaB (NF-kB) element. In contrast, Tax 703 activates the NF-kB element but does not affect CRE. The expression vectors for p65 and p50 were also used. The IκBα and IκBβ dominant-negative mutants of IκBα (IκKα, IκKβ, IKKa, IKKβ) and IκKγ (K44A), IκKβ, IκKβ, IkKα (1-305) and NF-kB-inducing kinase (IKKγ) were synthesized from 1 l of plasmid DNA using the In-Fusion HD cloning system (Clontech Laboratories, Palo Alto, CA). Various luciferase expression constructs containing the AID promoter fragment into a pGL3 basic vector were described previously (30, 34). The 0.9XκB construct was constructed by deletion of the NF-kB site of the pκB site of 0.9 (900 bp 5’ fragment only). Jurkat cells were transfected with the appropriate reporter and effector plasmids by electroporation. After 48 h, luciferase assays were performed with the dual luciferase assay system (Promega). Luciferase activities were normalized relative to the Renilla luciferase activity from pRL-TK.

Small interfering RNA

To repress p50 and p65, redesigned double-stranded small interfering RNAs (siRNAs; ON-TARGET plus SMART pool; Dharmacon, Lafayette, CO) were used. The control siRNA (Dharmacon) was used as a negative control. All siRNA transfections were performed using a MicroPorator MP100 (Digital Bio Technology, Seoul, Korea), pulsed twice at 1100 V for 30 ms.

Preparation of nuclear extracts and electrophoretic mobility shift assay

Nuclear proteins were extracted and transcription factors bound to specific DNA sequences were examined by electrophoretic mobility shift assay (EMSA) as described previously (31). The top strand sequence of the oligonucleotide probe or competitors are as follows: for the NF-κB element of the AID gene, 5’-GATGTCGTCGGAAGGGGAGCCCAACAGAAGA-3’; for the NF-κB element of the IL-2 receptor α chain gene, 5’-GATCCCGGGGAGGAACCTGCTGTT-3’; several NF-κB elements of the inducible nitric oxide synthase (iNOS) gene, 5’-TCGAGTGGATGACTCTCTCTC-3’ and for the AP-1 element of the IL-8 gene, 5’-GATCAGTGGATTACCTAGTTGT-3’. The above underlined sequences are the NF-κB and AP1-binding sites, respectively. To identify the transcription factors, in the DNA-protein complex shown by EMSA, we used antibodies for various NF-κB family proteins, including p50, p65, p50, c-Rel, p52 and RelB (Santa Cruz Biotechnology).

Immunoprecipitation and immunoblotting

To examine protein–protein interaction in MT-2 cells, whole-cell lysates were prepared from cells using lysis buffer (50 mM Tris, pH 8.0, 10 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.5% NP-40 and 1 mM phenylmethyl-sulfonyl fluoride). Whole-cell extract was incubated with 2 µg of antibodies specific for Bcl-3 and p50 or control rabbit IgG for 4 h at 4°C on a rotator. Following incubation with the antibodies, lysates were incubated with Protein G Sepharose 4 Fast Flow (GE Healthcare, Piscataway, NJ) for 16 h at 4°C on a rotator. After washing the beads three times in ice-cold lysis buffer and once in wash buffer (50 mM Tris, pH 8.0), the beads were suspended in sample buffer (1% sodium dodecyl sulfate, 100 mM dithiothreitol and 50 mM Tris, pH 8.0).
7.5). Before loading, the beads were boiled for 5 min, spun and the eluate was subjected to sodium dodecyl sulfate–polyacrylamide gels. Following transfer, blots were immunoblotted as in western analysis section. Mouse monoclonal antibody to p50 and rabbit polyclonal antibody to Bcl-3 (Santa Cruz Biotechnology) and rabbit polyclonal antibody to p50 (Cell Signaling Technology) were used for immunoprecipitation and western blot.

Results

AID expression in HTLV-1-infected T-cell lines and primary ATL cells

First, we examined the expression of Tax in various human T-cell lines (Figure 1A). Tax messenger RNA (mRNA) was highly expressed in HTLV-1-transformed T-cell lines (MT-2, MT-4 and C5/MJ) but not in ATL-derived T-cell lines, TL-OmI and ED-40515(-) cells. Tax protein was detected in all HTLV-1-transformed T-cell lines but not in any ATL-derived T-cell lines. We next examined the expression of AID at mRNA and protein levels in various human T-cell lines by RT–PCR and western blot analysis, respectively. As shown in Figure 1A, compared with uninfected T-cell lines, HTLV-1-transformed T-cell lines but not ATL-derived T-cell lines, expressed AID mRNA and protein (Figure 1A). APOBEC3 family, consisting of APOBEC3A-3H, consists of cellular proteins with cytidine deaminase activity that induces dC-to-dU mutations in minus-stranded DNA formed during RT to disable a broad range of retroviruses. APOBEC3G has been identified to potentiate the inhibition of human immunodeficiency virus type 1 (36). Expression of APOBEC3G mRNA was not associated with HTLV-1 infection (Figure 1A). Furthermore, we examined the mRNA expression of AID in primary ATL cells freshly isolated from acute-type patients and in PBMCs from normal subjects. Primary ATL cells from seven of nine patients expressed AID mRNA at significantly higher levels than normal PBMCs (Figure 1B). Tax protein was not expressed in all types of cells (data not shown). Phytohemagglutinin also induced AID mRNA expression (Figure 1C). To clarify whether AID expression is restricted to ATL cells, we examined AID mRNA expression in other lymphoid leukemia. However, no specific fragment could be amplified from B-ALL and T-ALL samples except for 1 B-ALL sample (Figure 1C). Immunohistochemical staining identified ATL cells positive for AID in the cytoplasm of all skin tissue (n = 10) and lymph node (n = 6) samples examined (Figure 1D), although the staining intensity varied from one specimen to another.

![Fig. 1. AID is consistently expressed in HTLV-1-infected T-cell lines and primary ATL cells. (A) Expression of AID, APOBEC3G and Tax in HTLV-1-infected T-cell lines. RT–PCR analysis was carried out for AID, APOBEC3G, Tax and β-actin (loading control). Western blot analysis was performed for AID, Tax and actin. Lt-4 monoclonal antibody detected a 40 kDa molecule (p40) in MT-4 and C5/MJ cells and p40 and p68 (a fusion between the envelope and the Tax-coding sequence) in MT-2 cells. Histograms indicate the relative density data of AID mRNA by densitometric analysis of the bands shown in the top panel normalized to β-actin mRNA (fourth panel). (B) RT–PCR analysis for AID expression in normal PBMCs and primary ATL cells. Normal PBMCs from healthy donors (n = 4) and freshly isolated primary ATL cells (>90% leukemic cells) from patients (n = 9) were examined as indicated. Histograms indicate the relative density data of AID mRNA obtained by densitometric analysis of the bands shown in the top panel normalized to β-actin mRNA (bottom). (C) RT–PCR analysis for AID expression in phytohemagglutinin-stimulated and -unstimulated PBMCs, B-ALL and T-ALL samples. Phytohemagglutinin–PBMC: normal PBMCs treated with phytohemagglutinin for 3 days. (D) Immunohistochemical staining of AID in ATL lymph nodes and skin lesions. Tissue sections from ATL lymph nodes (n = 6) and skin lesions (n = 10) were stained with anti-AID antibody. Tissue sections were counterstained using methyl green. Representative results from a single donor. The arrows indicate typical AID-positive tumor cells. Original magnification, ×400 and ×1200.](https://academic.oup.com/carcin/article-abstract/32/1/110/2477273)
In lymph node, these AID-positive tumor cells were varying in size (medium and large) and showed severe nuclear irregularity. In skin tissue, these AID-positive tumor cells were varying in size (small to large) and showed mild to moderate nuclear irregularity.

**AID expression during HTLV-1 infection**

To examine whether HTLV-1 infection induces AID expression in PBMCs, we cocultured PBMCs and MMC-treated HTLV-1-infected MT-2 cells. At 7 days after cocultivation, PBMCs were harvested for assessment for expression of HTLV-1 viral gene by RT–PCR. PBMCs cocultured with MMC-treated MT-2 cells expressed Tax mRNA (Figure 2A). Furthermore, AID expression levels increased in these cells in conjunction with induction of HTLV-1 gene. Since MT-2 cells were pretreated extensively with MMC, no discernible MT-2 cells were seen. Trypan blue staining confirmed that no MT-2 cells were viable. This assured that no MMC-treated MT-2 cells persisted in the PBMCs culture at the time of RNA isolation. Previously, the possibility that gene amplification was due to the contamination from residual MT-2 cells was excluded (37,38). Furthermore, AID expression levels did not increase in PBMCs cocultured with MMC-treated Jurkat cells and the supernatants of MT-2 cells had no substantial effects on PBMCs (data not shown). These results suggest that infection of PBMCs with HTLV-1 induces expression of AID. Similarly, cocultivation of the human T-cell line TY8-3 with MMC-treated MT-2 cells upregulated AID expression (Figure 2B). Thus, infection with HTLV-1 induces the expression of AID in both PBMCs and a T cell line.

**Tax-dependent expression of AID**

Tax gene product is the primary viral transactivator protein that modulates the expression of both viral and cellular genes. To examine whether Tax induces AID expression, we used JPX-9 cells, a Jurkat subline that carries the tax gene under the control of the metallothionein gene promoter (21). Treatment of these cells with CdCl₂ rapidly induced the expression of Tax mRNA (Figure 2C) as well as AID mRNA expression. In contrast, this treatment did not show any effect in Jurkat cells (Figure 2C).

As shown in Figure 1B, the primary ATL cells isolated from not all patients expressed AID. We further examined the coexpression of AID and Tax in ATL cells cultured for 3 days. Although circulating ATL cells freshly isolated from patients (ATL 93 and 100) hardly expressed Tax mRNA, the expression level markedly increased after 24–72 h of culture. CpG methylation of the HTLV-1 long terminal repeat plays a critical role in repression of viral gene expression in latently infected cells (39). This increased Tax mRNA expression might be due to reactivation of viral gene expression. AID mRNA was also expressed in primary ATL cells after 24–72 h of culture in parallel with Tax mRNA (Figure 2D). Taken together, these results further support the view that Tax induces AID.

To test the effect of Tax on AID expression at the transcriptional level, we performed luciferase reporter assays in Jurkat cells using an AID promoter-luciferase reporter plasmid (34). Cotransfection of an expression vector for Tax strongly activated AID promoter dose and time dependently in Jurkat cells (Figure 3A), indicating that Tax directly activates the AID promoter.

To narrow down the transactivation-relevant signaling pathways, Tax mutants M22 and 703 (29) were cotransfected along with the AID promoter construct, followed by determination of luciferase activities. Tax M22, which can activate CREB but is defective in NF-κB activation, did not activate the AID promoter. Tax 703, which can activate NF-κB but not CREB, failed to activate the AID promoter. However, Tax mutants M22 and 703 together activated the AID promoter, although the levels were less than wild-type Tax (Figure 3B). These results suggest that Tax activates the AID promoter in NF-κB and CREB-dependent manners.

We next examined whether Tax-mediated transactivation of AID gene expression involves signal transduction components in NF-κB.

---

**Fig. 2.** HTLV-1 and Tax induce AID expression. (A and B) Expression of HTLV-1 Tax and AID during HTLV-1 infection of normal PBMCs and TY8-3. Normal PBMCs and TY8-3 were cocultured with or without MMC-treated MT-2 cells. After cocultivation, cells were harvested, and the expression of the indicated genes was analyzed by RT–PCR. β-actin mRNA was used as a control. (C) Induction of AID mRNA expression by Tax. JPX-9 and Jurkat cells were treated with or without 20 μM of CdCl₂ for the indicated time periods. RT–PCR was carried out for AID, Tax and β-actin (loading control). (D) RT–PCR analysis for AID and Tax expression in PBMC samples from patients with ATL (ATL 93 and 100) without or with 24–72 h of culture.
Fig. 3. Tax transactivates the AID promoter through both NF-κB and CREB pathways. (A) Effect of Tax overexpression on AID promoter. Jurkat cells were transfected with increasing amounts of expression plasmid for HTLV-1 Tax along with 0.9P (5 μg) using electroporation. Cells were harvested 24 or 48 h after transfection, and luciferase activity was measured with a luminometer. (B) Tax activates the AID promoter by NF-κB and CREB. Jurkat cells were transfected with 0.9P (5 μg) together with the expression vector for HTLV-1 Tax (Tax WT), Tax M22, Tax 703 or empty vector (10 μg) alone or the combination of Tax M22 and Tax 703. (C) Functional effects of IkBα, IkBβ and IKK-β-dominant-interfering mutants and kinase-deficient IKKα, IKKβ and NF-κB-inducing kinase mutants on Tax-mediated activation of the AID promoter. Jurkat cells were transfected with 0.9P (5 μg) together with Tax (10 μg) and the indicated DN mutants or empty vector (5 μg). (D) Functional effects of CREB DN mutants on Tax-mediated activation of AID promoter. Jurkat cells were transfected with 0.9P (5 μg) together with Tax (10 μg) and KCREB, CREB133 or empty vector (5 μg). Cells were harvested 48 h after transfection, and luciferase activity was measured with a luminometer. The results are expressed as fold induction by Tax or Tax mutants relative to the vector alone. Data are mean ± SD of three independent transfection experiments.

activation. The dominant interfering mutants of IkBα, IkBβ and IKKγ and kinase-deficient mutants of IKKα, IKKβ and NF-κB-inducing kinase were tested for their ability to inhibit Tax-mediated transactivation of AID-driven reporter gene activity. The expression of these inhibitory mutants inhibited Tax-induced activation of AID promoter (Figure 3C), suggesting that signaling components involved in the activation of NF-κB are necessary for Tax transactivation of the AID promoter.

We also tested the potential role of CREB in modulating Tax-induced AID promoter activity. For this purpose, the dominant-negative CREB mutants KCREB and CREB133 were transfected into Jurkat cells with the AID promoter construct as well as an expression vector for Tax. KCREB contains a Ser-to-Ala mutation corresponding to amino acid 133, and this mutation blocks CREB phosphorylation, thus preventing transcription (40). Compared with transfections of an empty vector, KCREB- and CREB133-expressing Jurkat cells showed little Tax-mediated induction of the AID promoter (Figure 3D), suggesting that CREB is also required for Tax-mediated AID promoter activation.

To determine which site(s) of the AID gene promoter is responsible for the direct Tax-induced activation, we used plasmid constructs containing the 5′-flanking region and first intron. Two regions (CR1 and CR2) are present in the first intron and two E-boxes, the consensus sequence for E2A binding are present in the CR2 region (34). As shown in Figure 4A, 0.9P (promoter only), 0.9P-CR1+CR2 (promoter plus CR1 and CR2 fragments), 0.9P-CR1+CR2ΔE (promoter plus CR1 and CR2 fragments with deleted E-boxes) and 0.9P+I (promoter plus first intron) responded to Tax, suggesting the presence of a Tax-responsive element in the 5′-flanking region of the AID gene. To further delineate the Tax-responsive element within this region, we used various deletion constructs. As shown in Figure 4A, the 0.02P promoter lost the responsiveness to Tax, indicating that a Tax-responsive element may exist between −121 and −21 bp from the initiation site. We found one NF-κB-binding site, GGGAGGAGCC, which differed from the consensus sequence in two bases within this region and analyzed the promoter activity of the construct with deletion of this site (Figure 4B). The deletion reduced the response of the gene promoter to Tax activation. A residual transactivation by Tax hints to a minor contribution of other transcription factors including CREB. Thus, the NF-κB site seems to be the major element responsible for Tax-induced activation of AID gene.

HTLV-1 and Tax elicit NF-κB subunit binding to the AID NF-κB element

In the next step, we determined whether HTLV-1 infection induces NF-κB binding to the NF-κB element in the AID gene. EMSA was performed with double-stranded oligonucleotides representing the NF-κB element. Compared with the control uninfected T-cell lines and ATL-derived T cell lines, protein complexes bound to the NF-κB site were detected in nuclear extracts from all HTLV-1-transformed T-cell lines examined at high levels (Figure 5A, left). Furthermore, protein complexes bound to the NF-κB element were detected in nuclear extracts from primary ATL cells but not normal PBMCs (Figure 5B, left).

The specificity of DNA–protein complex formation was determined by competition studies with unlabeled competitors. As
expected, a 100-fold molar excess of 'cold' AID NF-κB double-stranded oligonucleotides effectively competed with the labeled probe and eliminated the binding of nuclear extracts from MT-2 cells and primary ATL cells (Figure 5A and B, right, lanes 1 and 2). An unlabeled IL-8 AP-1 probe could not compete with a labeled probe (Figure 5A and B, right, lanes 1 and 4). Interestingly, a consensus NF-κB site from the IL-2 receptor α chain promoter could not also compete with the probe (Figure 5A and B, right, lanes 1 and 3). The exact composition of the transcription factor DNA–protein complexes in HTLV-1-infected T cells was ascertained by supershift analysis. Supershift reactions performed using MT-2 nuclear extracts indicated that p50 was the predominant component of the NF-κB complexes in MT-2 cells (Figure 5A, right, lane 5).

Nuclear extracts from JPX-9 cells also showed that Tax expression alone elicited binding to the AID NF-κB probe (Figure 5C, top). Induction of Tax expression resulted in the formation of a complex with the AID probe within 4 h, and this complex was eliminated by competition with a 100-fold molar excess of unlabeled AID NF-κB oligonucleotides but not by an IL-2 receptor α chain promoter polynucleotide and an AP-1 (Figure 5C, bottom, lanes 2–5). These results suggest that HTLV-1 infection and Tax expression result in the binding of the NF-κB complex to the NF-κB element of the AID promoter and that this complex differed from the NF-κB complex bound to the consensus NF-κB site.

To examine whether this site can bind p50 protein, recombinant p50 protein was analyzed by EMSA. Incubation of AID NF-κB oligonucleotides with the recombinant p50 produced a specific retarded complex (Figure 5D, lane 1) that could be inhibited by preincubation with a 100-fold molar excess of unlabeled NF-κB oligonucleotides but not by AP-1 (Figure 5D, lanes 1–5). The specific retarded complexes formed with recombinant p50 was supershifted with p50 antibody but not with p65 antibody (Figure 5D, lanes 6 and 7), indicating that this site is capable of binding p50 protein.

Based on the above results, it was intriguing to examine whether exogenous expression of subunits of NF-κB activate the AID promoter. Introduction of the p50 but not p65 subunit activated the AID promoter (Figure 5E), suggesting that activation of AID promoter by Tax is mediated by NF-κB p50 pathway.

To provide further evidence for the role of p50 in the signal transduction pathway leading to Tax-induced AID expression, the use of siRNA to suppress p50 decreased the expression of AID mRNA (Figure 5F, left) and NF-κB DNA binding (Figure 5F, right). In contrast, p65 siRNA did not inhibit the expression of AID (Figure 5F, middle). Next, we examined the Tax-mediated AID expression with NF-κB signaling inhibitors. We found that the NF-κB inhibitory reagents, N-acetyl-L-leucyl-L-leucyl-L-norleucinal [a proteasome inhibitor; (41)] and Bay 11-7082 [an inhibitor of IκBα phosphorylation; (42)] significantly suppressed AID mRNA expression (Figure 5G). Taken together, these findings indicate that the Tax-induced AID expression in T cells is mediated mainly through the activation of NF-κB.

**HTLV-1-infected T cells express the transcriptional coactivator Bcl-3 that complexes with p50**

The p50 homodimer binds DNA in vitro, but lacks a transcriptional activation domain and, consequently, initiates a weak, if any, transcription. In fact, this NF-κB homodimer appears to function as a transcriptional inhibitor in some cell types by competing with p50/p65 for promoter binding (43). Countering this inhibitory effect in a variety of transcriptional complexes is the oncoprotein Bcl-3, which binds p50 homodimers and robustly coactivates transcription (44). Experiments were thus conducted to explore the potential role of Bcl-3 in p50 transcriptional coactivation in HTLV-1-infected T cells. RT–PCR and western blots demonstrated high expression levels of Bcl-3 mRNA and protein in HTLV-1-infected T-cell lines compared with
inhibitors on endogenous AID expression in an HTLV-1-infected T cell line. MT-2 cells were treated with either Bay 11-7082 (10 μM) or control siRNA. At 48 h after transfection, total RNA was isolated from each cell, and the expression levels of p50, p65, AID and endogenous p50 reduce the expression of AID mRNA and NF-κB factors to the NF-κB site in AID promoter. Nuclear extracts were subjected to EMSA with the AID NF-κB site (lane 2), a consensus NF-κB site from the IL-2 receptor α chain (IL-2αRα) promoter (lane 3) or an AP-1 site from the IL-8 promoter (lane 4). The indicated unlabeled oligonucleotides were incubated with nuclear extracts for 15 min before binding reactions. Nuclear extracts from MT-2 cells were also subjected to supershift assays with either no antibody (lane 1) or the indicated antibodies (Abs; lanes 5–9). The Abs were incubated with nuclear extracts for 45 min before binding reactions. Arrows: the specific complexes, arrowheads: the DNA binding complexes supershifted by Abs. (A) Tax-induced NF-κB-binding activity. Nuclear extracts from MT-2 cells treated with Bay 11-7082 (20 μM) for the indicated time periods were incubated with the labeled double-stranded oligonucleotides representing the AID NF-κB site (top). Nuclear extracts from JX9-9 cells treated with Bay 11-7082 (20 μM) for 4 h were subjected to competition analysis with a 100-fold molar excess of unlabeled double-stranded oligonucleotides representing the AID NF-κB site (lane 3), a consensus NF-κB site from the IL-2αRα promoter (lane 4) and an AP-1 site from the IL-8 promoter (lane 5). (B) Recombinant NF-κB subunit p50 was subjected to EMSA using the labeled double-stranded oligonucleotides representing the AID NF-κB site. Binding reaction was carried out in the presence of the indicated competitors (lanes 2–5). Lane 1, DNA complex in the absence of any competitor. In addition, incubation with antibody to p50 (lane 6) but not p65 (lane 7) supershifted the complex. Arrows: the specific complexes, arrowheads: the DNA-binding complexes supershifted by Abs. (E) Effect of overexpression of p50 and p65 on AID promoter. Jurkat cells were transfected with an expression plasmid for either p50 or p65 component of NF-κB (5 μg) along with either pGL3 basic or AID 0.9P (5 μg). Luciferase activity was analyzed, and the fold induction of p50 or p65 activity was calculated relative to the empty vector. Data are mean ± SD of three independent transfection experiments. (F) Suppression of endogenous p50 reduces the expression of AID mRNA and NF-κB DNA binding. MT-2 cells were transfected with either p50 or control siRNA and either p65 or control siRNA. At 48 h after transfection, total RNA was isolated from each cell, and the expression levels of p50, p65, and β-actin (loading control) mRNAs were measured by RT-PCR. Nuclear extracts were also isolated from each cell and subjected to EMSA with the AID NF-κB site (right). (G) Effects of NF-κB inhibitors on endogenous AID expression in an HTLV-1-infected T cell line. MT-2 cells were treated with either Bay 11-7082 (10 μM) or N-acetyl-L-leucyl-L-leucyl-l-norleucinal (20 μM) for the indicated time periods. Total RNA was isolated from each cell, and the AID mRNA expression level was measured by RT-PCR.

Fig. 5. p50 contributes to AID expression. (A and B) HTLV-1 infection is associated with binding of NF-κB factors to the NF-κB site in AID promoter. Nuclear extracts (5 μg) from control uninfected and HTLV-1-infected T-cell lines (A) and PBMCs from healthy volunteers and patients with ATL (B) were incubated with the labeled double-stranded oligonucleotides representing the AID NF-κB site in EMSA reactions. Nuclear extracts from MT-2 (A, right) and ATL (B, right) cells were subjected to competition analysis with a 100-fold molar excess of unlabeled double-stranded oligonucleotides representing the AID NF-κB site (lane 2), a consensus NF-κB site from the IL-2 receptor α chain (IL-2αRα) promoter (lane 3) or an AP-1 site from the IL-8 promoter (lane 4). The indicated unlabeled oligonucleotides were incubated with nuclear extracts for 15 min before binding reactions. Nuclear extracts from MT-2 cells were also subjected to supershift assays with either no antibody (lane 1) or the indicated antibodies (Abs; lanes 5–9). The Abs were incubated with nuclear extracts for 45 min before binding reactions. Arrows: the specific complexes, arrowheads: the DNA binding complexes supershifted by Abs. (C) Tax-induced NF-κB-binding activity. Nuclear extracts from JX9-9 cells treated with CdCl₂ (20 μM) for the indicated time periods were incubated with the labeled double-stranded oligonucleotides representing the AID NF-κB site (top). Nuclear extracts from JX9-9 cells treated with CdCl₂ (20 μM) for 4 h were subjected to competition analysis with a 100-fold molar excess of unlabeled double-stranded oligonucleotides representing the AID NF-κB site (lane 3), a consensus NF-κB site from the IL-2αRα promoter (lane 4) and an AP-1 site from the IL-8 promoter (lane 5). (D) Recombinant NF-κB subunit p50 was subjected to EMSA using the labeled double-stranded oligonucleotides representing the AID NF-κB site. Binding reaction was carried out in the presence of the indicated competitors (lanes 2–5). Lane 1, DNA complex in the absence of any competitor. In addition, incubation with antibody to p50 (lane 6) but not p65 (lane 7) supershifted the complex. Arrows: the specific complexes, arrowheads: the DNA-binding complexes supershifted by Abs. (E) Effect of overexpression of p50 and p65 on AID promoter. Jurkat cells were transfected with an expression plasmid for either p50 or p65 component of NF-κB (5 μg) along with either pGL3 basic or AID 0.9P (5 μg). Luciferase activity was analyzed, and the fold induction of p50 or p65 activity was calculated relative to the empty vector. Data are mean ± SD of three independent transfection experiments. (F) Suppression of endogenous p50 reduces the expression of AID mRNA and NF-κB DNA binding. MT-2 cells were transfected with either p50 or control siRNA and either p65 or control siRNA. At 48 h after transfection, total RNA was isolated from each cell, and the expression levels of p50, p65, and β-actin (loading control) mRNAs were measured by RT-PCR. Nuclear extracts were also isolated from each cell and subjected to EMSA with the AID NF-κB site (right). (G) Effects of NF-κB inhibitors on endogenous AID expression in an HTLV-1-infected T cell line. MT-2 cells were treated with either Bay 11-7082 (10 μM) or N-acetyl-L-leucyl-L-leucyl-l-norleucinal (20 μM) for the indicated time periods. Total RNA was isolated from each cell, and the AID mRNA expression level was measured by RT-PCR.

Finally, we examined the expression levels of AID, Bcl-3 and Tax mRNAs in primary ATL cells. As shown in Figure 6E, ATL cells constitutively expressed Bcl-3 mRNA, but ATL cells from not all patients expressed AID mRNA. AID mRNA expression did not correlate with that of Tax. Although we could not examine protein complexes bound to the NF-κB site in these cells, activation of p50/Bcl-3 complexes might explain the expression of AID in primary ATL cells.

Discussion

The involvement of AID in the progression of leukemia has been suggested based on the correlation between AID expression and poor prognosis of patient with B-cell chronic lymphocytic leukemia (45,46). However, a simple correlation does not tell us whether AID expression is the cause or the result of leukemia. On the other hand, a causal relationship was suggested in a study of AID transgenic mice
AID expression in ATL

(13). These mice spontaneously and frequently develop T-cell lymphoma and lung microadenoma in association with high mutation frequencies. The reason for T cells and the lung epithelium to be the targets of cancer formation in AID transgenic mice, despite the ubiquitous expression of AID is unknown at present. Nonetheless, the fact that aberrant AID expression in mice led to T-cell lymphoma suggests that AID could be the cause of T-cell neoplasms.

In the present study, we examined AID expression in HTLV-1-infected T-cell lines and primary ATL cells and compared the expression level with that in uninfected T-cell lines from T-ALL patients and normal PBMCs. The results showed that HTLV-1-transformed T-cell lines (3/3) and ATL cells from 14 of 18 patients expressed significantly higher levels of AID than did any other cells. Furthermore, the cytoplasmic AID expression was confirmed by immunohistochemistry in primary ATL cells invading skin tissues (10/10) and lymph nodes (6/6). However, AID expression was negative in T-ALL (2/2) and positive in only one sample in six B-ALL ones. The G to A mutations (e.g. Arg273His and Gly245Asp) of the p53 gene were reported in primary ATL cells (47), suggesting that AID could target the p53 gene in ATL.

One important finding of this study was that HTLV-1 and viral transforming protein Tax triggered aberrant AID expression in T cells. Indeed, AID expression correlated with that of Tax in T-cell lines. Furthermore, we identified AID as a target gene of the NF-κB and CREB activation pathways in T cells. Since we did not identify the putative binding elements for CREB between −121 and −21 bp from AID promoter sequence analyses, CREB is probably not directly involved in Tax-induced AID expression. Alternatively, these results suggest the involvement of a Tax-response element different from the known CRE site. The presence of p50 but not p65 in the NF-κB DNA complexes of the AID promoter in HTLV-1-infected T cells is an intriguing finding. This NF-κB subtype may be involved in the competitive transcriptional inhibition of p50/p65, a function frequently attributed to the p50 homodimer (43). However, the expression of the transcriptional coactivator Bcl-3 in the HTLV-1-infected T-cell line, and thecomplexing of Bcl-3 with p50, provides preliminary evidence suggesting that the p50 homodimers may be transcriptionally active. The oncprotein Bcl-3 is a member of the IkB family of NF-κB binding proteins. The BCL3 gene, located on chromosome 19, is translocated intact and is activated in cases of t(14;19)(q32.3;q13.2) B-cell chronic lymphocytic leukemia (48). Bcl-3 has been implicated also in the pathogenesis of nasopharyngeal carcinoma, breast cancer and a variety of lymphomas, all cases giving evidence for coactivation of NF-κB p50 by Bcl-3 as the transforming event (44,49–51). Bcl-3 expression and its interaction with NF-κB p50 have not been previously reported in HTLV-1-infected T cells, although Tax is known to associate with Bcl-3 and induce Bcl-3 expression (52,53). It is interesting to speculate that Bcl-3/p50 complexes may be important in the transcription of mutation inducing gene AID. Tax induces Bcl-3 expression through stimulation of the NF-κB pathway (53), suggesting that AID gene is the primary and secondary target of Tax-induced NF-κB activation. Bcl-3 also functions as a repressor of transcription from the HTLV-1 long terminal repeat (52,53). Bcl-3 may promote somatic mutation frequency through the induction of AID and may attenuate virus production, facilitating immune evasion.

It is known that after the early phase of HTLV-1 infection, Tax expression is repressed in vivo, probably due to immune surveillance. Tax-negative ATL-derived T-cell lines, TL-Omi and ED-40515(-), did not express AID, but primary ATL cells expressed AID. Interestingly, Tax-expressing cells and primary ATL cells had higher levels of the protein complexes bound to the NF-κB element in the AID promoter compared with Tax-negative ATL-derived T-cell lines. Since the expression of Tax in some primary ATL cells was only detected by RT–PCR (Figure 6E), a trace amount of Tax seems to be sufficient for

Fig. 6. HTLV-1-infected T cells express Bcl-3/p50 complexes. (A) Expression of Bcl-3 in HTLV-1-infected T-cell lines. RT–PCR analysis was carried out for Bcl-3 and β-actin (loading control). Western blot analysis was performed for Bcl-3 and actin. (B) Induction of Bcl-3 expression by Tax. JPX-9 cells were treated with or without 20 μM of CdCl2 for the indicated time periods. RT–PCR was carried out for Bcl-3 and β-actin (loading control). Western blot analysis was also performed for Tax, Bcl-3 and actin (loading control). (C) Immunofluorescence images of MT-2 cells for p50 (Alexa Fluor 488; green), Bcl-3 (Alexa Fluor 546; red) and nuclei (Hoechst 33342; blue). p50 colocalizes with Bcl-3 in situ in the nuclei of MT-2 cells; DIC, differential interference contrast. (D) Cell lysates from MT-2 cells were used for immunoprecipitation with anti-Bcl-3 or anti-p50 antibody followed by immunoblotting with anti-p50 or anti-Bcl-3 antibody. The expression level of p50 or Bcl-3 was detected (input). (E) RT–PCR analysis for AID, Bcl-3 and Tax expression in primary ATL cells.
the induction of AID in ATL cells. However, Tax-negative ATL cells (ATL 85 and 94) expressed AID mRNA at the relatively high levels. Alternatively, NF-κB activation might be necessary for the constitutive expression of AID. It is thus reasonable to assume that transactivation by Tax is not the only mechanism underlying over-expression of AID in ATL cells.

In conclusion, our findings provide the first evidence that AID is induced in response to Tax via the Bcl-3/NF-κB signaling pathway and might be involved in the development of ATL. However, there is room for further investigation to determine whether aberrant AID expression could contribute to the accumulation of genomic mutations in T cells. Recently, induction of reactive oxygen species by Tax has been reported to elicit DNA damage (54). Initiation of DNA double-strand break formation by AID requires reactive oxygen species intermediates, such as hydroxyl radicals (55). Tax could make structural DNA changes both through enzymes such as AID and through reactive oxygen species. Understanding the mechanisms of AID upregulation in T cells might provide a new strategy to prevent the development and progression of ATL. The constitutive expression of mutator enzyme AID in ATL cells can be speculated to result from mutations induced by the Tax-activated AID and/or other Tax-associated mutagenic mechanisms during the pre-leukemic stage.

Funding

This work was supported in part by Japan Leukemia Research Fund to NM and Osaka Cancer Research Foundation to N.M.

Acknowledgements

We thank the many patients with ATL and ALL and the control subjects who donated samples for these studies. We acknowledge Drs Shigeki Sawada and Tetsuro Nakazato and Mr Yukimasa Shibata for the expert technical assistance. We thank Drs Taeko Okudaira, Jun-Nosuke Uchihara, Naoya Taira, Kazuiku Ohshiro, Takehiro Matsuda, Hiroshi Uezato and Koichi Ohshima for providing patient samples. We thank Drs Kayoko Matsumoto, Dean W. Ballard, Romas Gelezianus and Kuan-Teh Jeang for providing expression vectors for Tax and its mutants; for IκBα and IκBβ-dominant-negative mutants; for NF-κB-inducing kinase, IKKα and IKKβ-dominant-negative mutants and for IKKγ-dominant-negative mutant. We thank Dr Masataka Nakamura for providing JXP-9, Dr Michiyuki Maeda for providing ED-40515(1) and Fujisaki Cell Center, Hayashibara Biomedical Laboratories (Okayama, Japan) for providing the C57Mj cell line. We also thank Dr Yuetsu Tanaka for providing Tax monoclonal antibody. Recombinant human IL-2 was kindly provided by Takeda Chemical Industries (Osaka, Japan).

Conflict of Interest Statement: None declared.

References


Received May 9, 2010; revised October 10, 2010; accepted October 19, 2010