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Possible involvement of aryl hydrocarbon receptor (AhR) in adult T-cell leukemia (ATL) leukemogenesis: constitutive activation of AhR in ATL

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Abstract

Human T-cell leukemia virus type 1 is the etiologic agent of adult T-cell leukemia (ATL), although the precise mechanism involved in the transformation process has not yet been defined. The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that can influence cell proliferation and differentiation. We investigated the expression and activation of AhR in ATL. RT-PCR and Western blot analyses showed high expression levels of AhR in ATL cell lines. The elevated expression of AhR was in part attributable to the action of the viral transactivator protein, Tax. Interestingly, activation of the AhR was found in ATL cell lines in the absence of apparent exogenous ligands. Importantly, the increased expression and activation of AhR were also observed in some primary ATL cells. To our best knowledge, this is the first report to show the lymphoid malignancy having constitutive activation of AhR. A possible link between increased AhR expression and leukemogenesis in ATL is discussed.

Keywords: Adult T-cell leukemia; Aryl hydrocarbon receptor

Adult T-cell leukemia (ATL) is defined as a mature $CD4^+$ T-cell leukemia [1] that is caused by human T-cell leukemia virus type 1 (HTLV-1) [2,3]. The precise mechanism of the transformation and leukemogenesis remains unclear, however, a 40 kDa nuclear oncoprotein termed Tax is thought to be responsible for these processes. Tax has been shown to activate the expression of numerous cellular genes involved in T-cell activation and growth through several distinct transcription factors, such as Rel/nuclear factor-κB (Rel/NF-κB), AP-1, and serum response factor [4–6]. Some of the genes that can be transactivated by Tax are also constitutively expressed in primary ATL cells, however, primary ATL cells are known to express Tax at low levels, indicating that there is another mechanism independent of Tax underlying the overexpression of several cellular genes in primary ATL cells [7].

The aryl hydrocarbon receptor (AhR) is a basic helix-loop-helix containing transcription factor which mediates gene expression in a ligand-dependent manner [8,9]. To date, a variety of coplanar aromatic molecules, such as several flavonoids, polycyclic aromatic hydrocarbons, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), have been identified as exogenous AhR ligands. In the absence of ligand AhR resides in the cytoplasm complexed with two heat shock protein 90 molecules and an immunophilin-like molecule. Following ligand binding the AhR translocates to the nucleus where it complexes with the aryl hydrocarbon nuclear translocator (ARNT) protein.

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The heterodimeric AhR–ARNT complexes recognize and bind to consensus sequences called AhRE in the regulatory domain of numerous genes involved in the metabolism of xenobiotics including cytochromes P-450 (CYP1A1, CYP1A2, and CYP1B1) and NAD(P)H-quinone oxidoreductase, and in conjunction with a series of coactivating proteins, stimulate target gene transcription [9,10]. An endogenous AhR ligand has been suggested from studies using cells in culture, where the induction of AhR-dependent gene expression has been observed in the absence of added exogenous ligand [11], and/or those investigating the occurrence of developmental defects in AhR knockout mice [12]. In addition, transcription of plasminogen activator inhibitor II, interleukin-1β, and protooncogenes c-*jun* and *jun* D can be activated by the AhR–ARNT complex [13,14]. Furthermore, AhR has been shown to influence cell cycle and differentiation [11,15], indicating that AhR contributes not only to the regulation of xenobiotic metabolism but also the maintenance of cell homeostatic functions.

Here, we investigated the expression and activation of AhR in ATL cell lines and primary ATL cells. We show that ATL cell lines have elevated expression of AhR. We further show that the elevated expression of AhR is at least in part attributable to the HTLV-1 transactivator, Tax. We also provide evidence that the AhR is activated in ATL cell lines in the absence of added exogenous ligands. Importantly, AhR is also up-regulated and activated in some primary ATL cells. These findings will be discussed in the context of leukemogenesis of ATL.

Materials and methods

Cell lines. Cell lines KK1, SO4, and ST1 are interleukin 2 (IL-2)-dependent T-cell lines of true ATL cell origin, as confirmed by the concordance of the integration site(s) of the HTLV-I proviral genome and/or the T-cell receptor β-chain gene rearrangement profiles with those of the respective original leukemia cells [16,17]. These ATL cell lines were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) and 0.25 U/ml recombinant-human IL-2 (provided by Takeda Chemical Industries, Osaka, Japan). The Jurkat human T-cell leukemia cells and HL60 human promyelocytic leukemia cells were grown in RPMI 1640 containing 10% FBS. JPX-9 (a subclone of Jurkat cells expressing Tax wild-type under the control of the metallothionein promoter [18]) was grown in RPMI 1640 containing 10% FBS. All cells were maintained at 37 °C in a 5% CO_2–95% air atmosphere.

Clinical specimens. Leukemic cells from 10 patients with acute-type ATL were analyzed. The diagnosis of ATL was based on clinical features, hematological findings, and the presence of anti-HTLV-1 antibodies in patient sera. Monoclonal HTLV-1 provirus integration into the DNA of leukemic cells was confirmed by Southern blot hybridization. Peripheral blood mononuclear cells (PBMCs) from healthy volunteers or patients with ATL were collected by Ficoll–Hypaque gradient and used as normal mononuclear cells and primary ATL cells, respectively. Morphological and marker analyses indicated that the proportions of ATL cells in patients' PBMC ranged from 80% to 95% at the time of analysis. All materials were obtained after informed consent.

RT-PCR. Total cellular RNA was extracted from each cell line, normal PBMCs, and primary ATL cells using ISOGEN kits (Nippon GENE, Toyama, Japan) and treated with DNase (Message CleanTM Kit; GenHunter, Nashville, TN, USA) to remove contaminating DNA. RT-PCR was performed according to manufacturer's directions (GeneAmp RNA PCR Kit; Perkin–Elmer, Foster, CA, USA). One microgram RNA was used for cDNA synthesis, which was carried out employing the Moloney murine leukemia virus RT (2.5 U/μl) and 2.5 μM oligo$(dT)_{16}$ as primer (total 20 μl) for 20 min at 42 °C. The reaction was stopped by heating the sample to 99 °C for 5 min. Aliquots (1 μl) of c-DNA were amplified in a 50-μl final volume with the specific primers for AhR, ARNT, CYP1A1, and β-actin as a control for successful c-DNA synthesis. The PCR primers used for AhR, ARNT, CYP1A1, and β-actin were 5′-TACTGAAGCAGAGCTGTGCA-3′ (forward)–5′-CTCATACAACACAGCTTCTCC-3′ (reverse; [19], 315 bp), 5′-CGGAACAAGATGACAGCCTAC-3′ (forward)–5′-ACAGAAAGCCATCTGCTGCC-3′ (reverse; [20], 226 bp), 5′-TTCA TCCCTATTCTTCGCTAC-3′ (forward)–5′-TCCATCAGCAT CT ATGTGGC-3′ (reverse; [21], 1073 bp), and 5′-TCATCACCATTGG-CA ATGAG-3′ (forward)–5′-CAGTGTGTTGGCGTACAG GT-3′ (reverse, 154 bp), respectively. The reaction was performed using *Taq* DNA polymerase (Qiagen, Tokyo, Japan), and 24–36 cycles of 94 °C 45 s, 62 °C 30 s, and 72 °C 30 s for AhR, 30 cycles of 94 °C 1 min, 65 °C 1 min, and 72 °C 1 min for ARNT, 30–39 cycles of 94 °C 1 min, 64 °C 1 min, and 72 °C 1.5 min for CYP1A1, and 24 cycles of 95 °C 1 min, 54 °C 1 min, and 72 °C 1 min for β-actin. The PCR products were separated on 2% agarose gels, stained with ethidium bromide, and visualized under UV light.

Real-time quantitative polymerase chain reaction. Real-time q-PCR was performed using the LightCycler Technology (Roche Diagnostics, Mannheim, Germany). For Tax mRNA amplification, 1 μL cDNA was added in a 20-μL final volume to 2 μL Mastermix (LightCycler DNA Master hybridization probes; Roche Diagnostics), $MgCl_2$ solution (2.4 mM), forward and reverse oligonucleotide primers (0.5 μM), and fluorescent hybridization probes (0.2 μM). The PCR program includes 50 cycles (95 °C, 10 s)/(64 °C, 5 s)/(72 °C, 10 s). Primers and probes used for Tax mRNA reverse-transcribed amplification were as follows: forward primer, 5′-CCCCCTTCCGAAATGGAT-3′; reverse primer, 5′-CCGGGGTGGCAAAAAATC-3′; FITC-probe, 5′-GAGG GTGTACAGGTTTTGGGGC-3′; and Red640-probe, 5′-GGAGTCC GGGGTCTGGAAAAGA-3′.

Detection of the PCR product was based on fluorescence resonance energy transfer between the fluorophores. Cycle threshold (C_T) was defined as the cycle number at which a significant increase in the fluorescence signal was first detected. Using a serial dilution of plasmids (10–10^7 molecules per reaction) of external standard made from a clone in Tax-PCR-fragment inserted in pGEM-T Easy Vector (Promega, Madison, WI, USA), the standard curve was generated between the C_T value and the logarithm of the starting copy number of plasmids. Quantification of the unknown samples was performed by the LightCycler software. The results were expressed initially as the number of target molecules/μL cDNA. To standardize the results for variability in RNA and cDNA quantity and quality, we quantified total GAPDH transcripts in each sample as internal control. GAPDH mRNA amplification was done under almost the same conditions for survivin RT-PCR except for $MgCl_2$ concentrations (4.0 mM). The PCR program includes 40 cycles (95 °C, 10 s)/(60 °C, 5 s)/(72 °C, 10 s). Primers and probes used for GAPDH mRNA reverse-transcribed amplification were as follows: forward primer, 5′-TGAACGGG AAGCTCACTGG-3′; reverse primer, 5′-TCCACCCTGTTGCTGT A-3′; FITC-probe, 5′-TCAACAGCGACACCCACTCCT-3′; and Red640-probe, 5′-CACCTTTGACGCTGGGGCT-3′. The GAPDH standard curve was generated by the same methods for Tax. Normalized levels of Tax transcripts were calculated as the ratio between the amounts of survivin transcripts and the GAPDH transcripts.

Western blotting. For preparation of total cell lysates, cell pellets (1×10^6 cells) washed with PBS were suspended in 100 μl of 1× SDS–

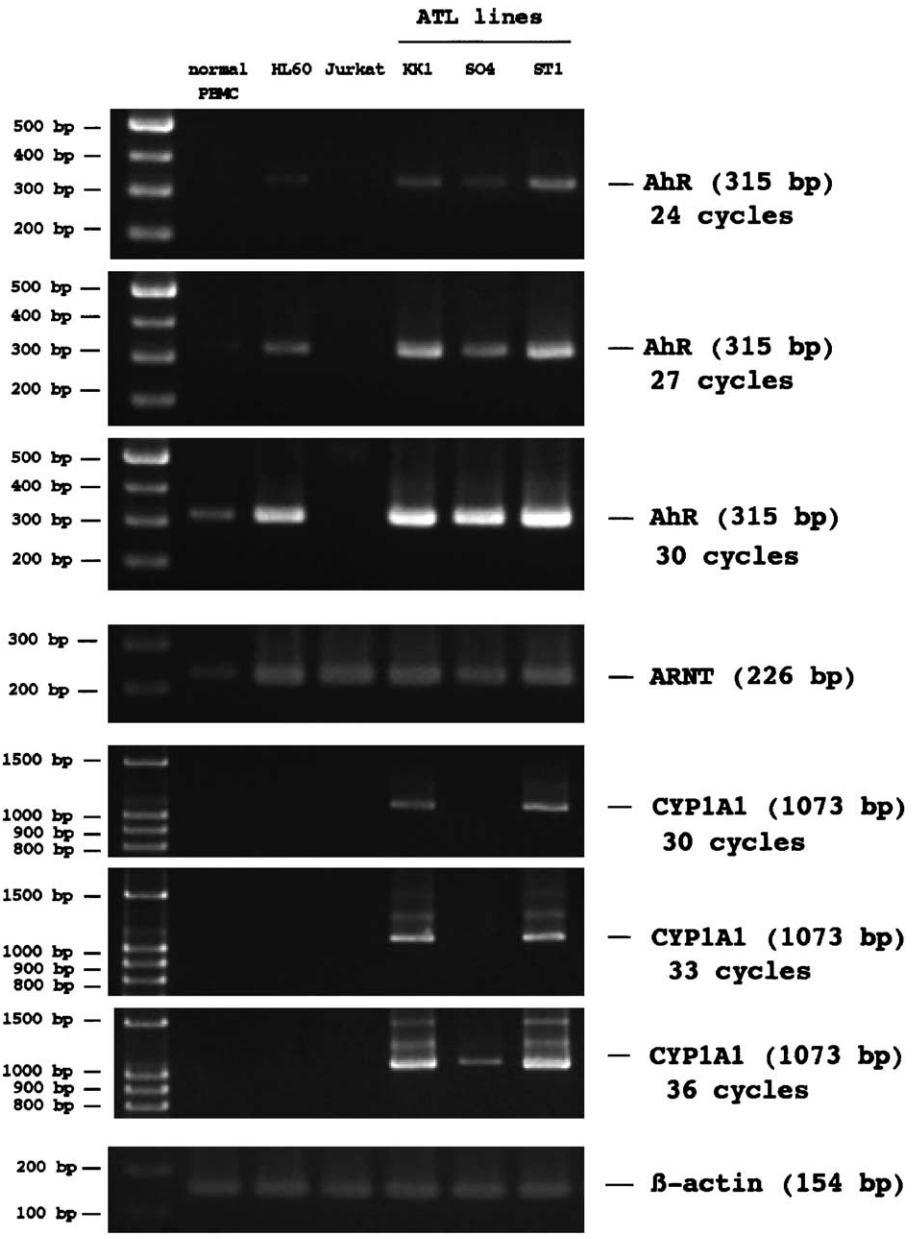

Fig. 1. Expression of AhR, ARNT, and CYP1A1 transcripts in ATL cell lines. Total RNA samples were prepared from ATL cell lines (KK1, SO4, and ST1 cells) as well as normal PBMCs. HL60 cells (a promyelocytic leukemia line) and Jurkat cells (a mature T-cell leukemia line) were used as a positive and negative control for AhR expression, respectively. RT-PCR analysis of these samples was performed using primers encoding AhR, ARNT, CYP1A1, and β-actin (an internal control).

PAGE sample buffer (50 mM Tris buffer, pH 6.8, 2% SDS, 10% glycerol, 5% of 2-mercaptoethanol, and 0.01% bromophenol blue) and boiled for 5 min. Nuclear and cytoplasmic fractions were prepared using a kit (Nuclear Extract Kit; Active Motif, Carlsbad, CA, USA) according to the manual. Then 10-μl aliquots of these total cell lysates, nuclear and cytoplasmic extracts were subjected to SDS–PAGE on a 7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Clear Blot Membrane-p; ATTO, Tokyo, Japan). AhR bound to the membrane was detected using an immunoassay kit (The Protein Detector Western Blot Kit BCIP/NBT System; KPL, Gaithersburg, MD, USA). Following transfer, the membrane was blocked in blocking buffer (included in the kit) for 1 h at room temperature. After the membrane had been washed with PBS, it was incubated with 5.9 μg/ml of a polyclonal goat anti-AhR antibody (Anti-Ah Receptor (AHR) Polyclonal; Novus Biologicals, Littleton, CO, USA) in the blocking buffer for 1 h at room temperature. The membrane was then washed in wash solution (included in the kit). After immunoblots were incubated with 0.4 μg/ml of an anti-goat IgG antibody conjugated with alkaline phosphatase (KPL, Gaithersburg, MD, USA) for 1 h at room temperature and washed with the wash solution, the immunoreacted proteins were visualized by addition of BCIP/NBT substrate (included in the kit). When a suitable color intensity was observed, the reaction was stopped by immersing the membrane in reagent quality water for 1–2 min. The same total cell lysates were also examined by 7.5% SDS–PAGE and visualized with Coomassie blue staining to confirm loading efficiency. Anti-lamin B (Oncogene Research Products, Cambridge, MA, USA) and anti-β-tubulin (Boehringer–Mannheim, Mannheim, Germany) antibodies were used to examine the cell fraction efficiency.

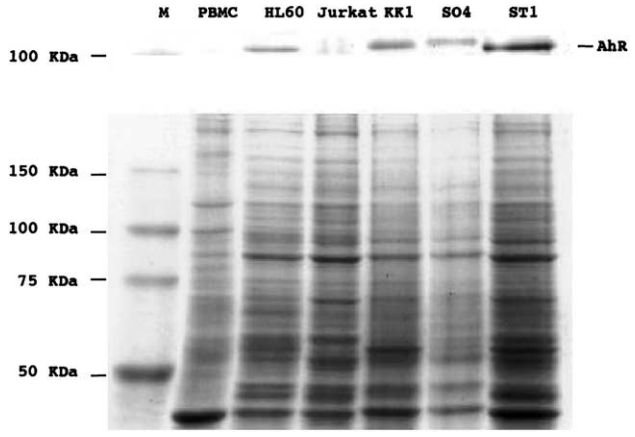

Fig. 2. Expression of AhR protein in ATL cell lines. Whole cell extracts were prepared from the indicated cell lines as well as normal PBMC, and analyzed by Western blotting (top) with anti-AhR antibody and by Coomassie blue staining after SDS–PAGE (bottom) showing the loading efficiency. Molecular mass was determined by the marker run in parallel.

Results and discussion

No lymphoid cell line expressing high levels of AhR is known as yet [19–23]. In contrast, AhR has been shown to be up-regulated in some acute myeloid leukemia (AML) cell lines and primary AML cells [19–22, 24], indicating that the AhR–ARNT heterodimer may play an important role in leukemogenesis in some AMLs. To investigate the possibility of whether the AhR–ARNT heterodimer is involved in leukemogenesis in ATL, we first analyzed the mRNA expression level of AhR and ARNT in ATL cell lines (KK1, SO4, and ST1) using RT-PCR as well as a promyeloid cell line HL60 (a known positive control for AhR-expression [19,20]) and a T-lymphoid cell line Jurkat (a known negative control for AhR-expression [19,21]). As shown in Fig. 1, we detected AhR mRNA in all the ATL cell lines examined and HL60 cells, but not in normal PBMCs and Jurkat cells at 24 PCR cycles. In normal PBMCs, AhR was detectable after 27 PCR cycles (Figs. 1 and 5). AhR has been reported to predominantly express in the monocyte fraction from normal blood cells [20]. Since PBMCs consist of a major lymphocyte fraction and a minor monocyte fraction, the AhR expression detected in normal PBMCs seems mainly to result from the minor monocyte fraction. In contrast, AhR was not detectable in Jurkat cells even after 30 PCR cycles and this observation is in keeping with the results of previous reports [19,21]. The expression of AhR protein was also confirmed by Western blot analysis (Fig. 2) and there was good qualitative agreement between the Western blot and the above results of RT-PCR at 24 cycles.

ARNT, an AhR partner cofactor, was detected in all cell lines and somewhat weaker in normal PBMCs (Figs. 1 and 5).

These findings demonstrate that ATL lines express high levels of AhR. To the best of our knowledge, this is the first report to show lymphoid cell lines expressing high levels of AhR.

ATL is a malignancy of peripheral $CD4^+$ T-cells initiated by infection with the HTLV-1. HTLV-1 Tax has been reported to affect the expression of numerous cellular genes. To investigate whether Tax affected AhR-

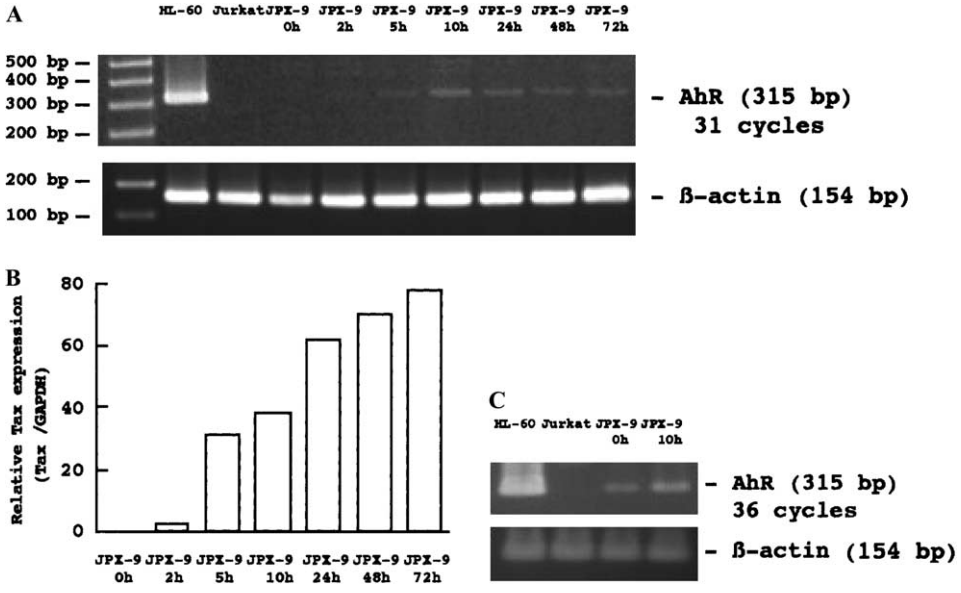

Fig. 3. Induction kinetics of the AhR gene in JPX-9 cells treated with $CdCl_2$. Total RNA samples were prepared from HL60, Jurkat, and $CdCl_2$-treated JPX-9 cells at the indicated time points (0–72 h). The expression of AhR and Tax in the extracted RNA was analyzed by RT-PCR (A, 31 cycles; C, 36 cycles) and real-time quantitative PCR (B), respectively. β-Actin served as an internal control in the RT-PCR procedure (A). Normalized levels of Tax transcripts were calculated as the ratio between the amount of Tax transcripts and GAPDH transcripts (B).

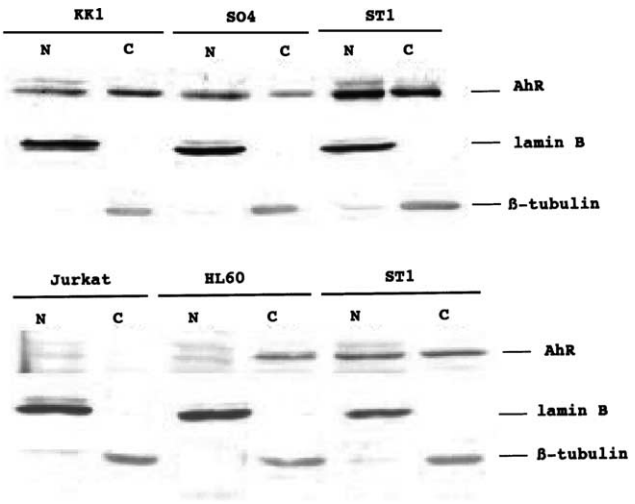

Fig. 4. Western blot analysis of cell fractions with anti-AhR antibody. ATL cell lines (KK1, SO4, and ST1 cells) were fractionated and reacted with anti-AhR antibody (top). Jurkat cells and HL60 cells were also fractionated and reacted with anti-AhR antibody as well as ST1 cells as a positive control (bottom). Anti-lamin B and anti-β-tubulin antibodies were used to examine cell fractionation. N, nuclear fractions; C, cytoplasmic fractions.

mRNA expression, we used JPX-9, which stably carries the Tax expression plasmid, pMAX-neo, in which the expression of Tax is inducible by the addition of $CdCl_2$. The level of expression of Tax mRNA in these cells was determined by real-time q-PCR and the expression of AhR-mRNA was assayed by RT-PCR. As shown in Fig. 3B, the addition of $CdCl_2$ (20 μM) to the culture medium of JPX-9 cells induced the expression of Tax within 5 h, which persisted until 72 h after treatment. Interestingly, a concomitant increase in the expression of AhR-mRNA within 5 h of treatment with $CdCl_2$ was observed in JPX-9 cells (Fig. 3A) after 31 PCR cycles. These results indicate that Tax itself is capable of causing elevated expression of the AhR-mRNA in Jurkat T-lymphoid cells and it may be in part responsible for the high expression of AhR in ATL cell lines. In addition, the expression of AhR-mRNA was found to be detectable even in $CdCl_2$-untreated JPX-9 cells (JPX-9 0 h) after 36 PCR cycles. The expression level in JPX-9 (0 h) was lower than that in JPX-9 (10 h) in this condition. With 36 PCR cycles, it remained negative in Jurkat cells (Fig. 3C). Since a low level of constitutive Tax expression has been known to leak from the metallothionein promoter even in the JPX-9 cells in the absence of $CdCl_2$ treatment, this result seems to further support a possible participation of Tax on the expression of AhR.

AhR has been defined and characterized according to its ability to mediate biological responses to exogenous ligands, such as the synthetic environmental contaminant TCDD. The natural ligand(s) for AhR is unknown, although some ligands have been suggested from studies using cells in culture, where induction of AhR-dependent gene expression has been observed in the absence of added exogenous ligand. The best characterized response to ligand-activated AhR is the transcriptional induction of the CYP1A1 gene. To determine whether activated AhR–ARNT complexes were present in unstimulated ATL cell lines, we first examined the CYP1A1-mRNA expression in ATL cell lines in the absence of added exogenous ligand by RT-PCR as well as in the HL60 and Jurkat cells. As shown in Fig. 1, CYP1A1-mRNA was undetectable in normal PBMCs and the Jurkat cells even after 36 PCR cycles. The CYP1A1-mRNA was also undetectable in HL60 cells regardless of its AhR-producing ability. In contrast, the CYP1A1-mRNA was apparently detectable in all the ATL cell lines examined after 30 (KK1 and ST1) or 36

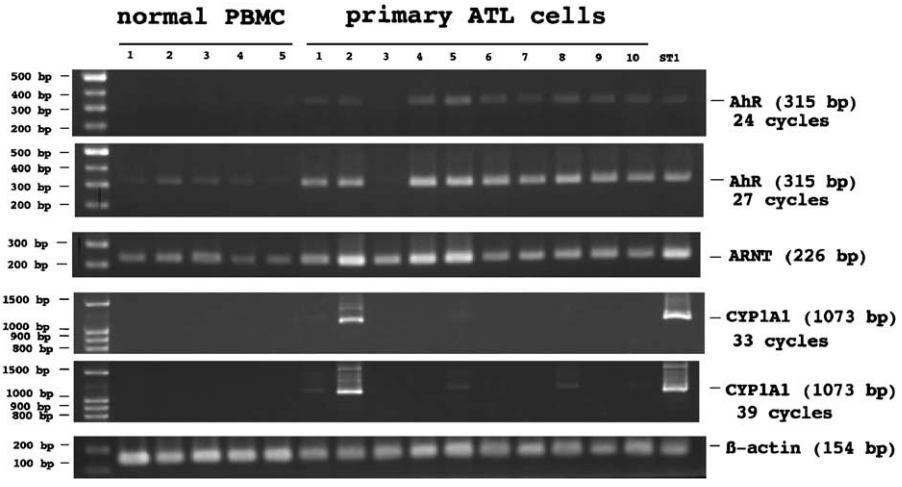

Fig. 5. Expression of AhR, ARNT, and CYP1A1 transcripts in primary ATL cells. Total RNA samples were prepared from primary acute ATL cells as well as normal PBMCs. RT-PCR analysis of these samples was performed using primers encoding AhR, ARNT, CYP1A1, and β-actin (an internal control).

(SO4) PCR cycles. These results suggested that AhR was naturally activated in unstimulated ATL cell lines but not in HL60 cells. To test this hypothesis, we further examined the AhR-localization in these cell lines because activated AhR translocates from the cytoplasm into the nucleus. In good agreement with the RT-PCR data, anti-AhR-Western blotting of the cell fractions revealed that the AhR was found to be localized in both the nucleus and cytoplasm in all the ATL cell lines (Fig. 4, top) but was localized in the cytoplasm alone in HL60 cells (Fig. 4, bottom), thus supporting our hypothesis and indicating the existence of endogenous AhR-ligand(s) in ATL cell lines.

To assess the relevance of our findings in clinical specimens, we analyzed the levels of expression of AhR, ARNT, and CYP1A1 in 10 primary ATL cells using RT-PCR as well as five additional normal PBMCs (see Fig. 5). Compared with normal PBMCs, the expression levels of AhR-mRNA were up-regulated in 9 of 10 primary ATL cells; these levels were either similar to or greater than that observed in ST1 expressing AhR protein. CYP1A1-mRNA was found to be clearly positive in 1 of 10 primary ATL cells after 33 cycles and was newly positive in three patient specimens after 39 cycles. With 39 cycles, it remained negative in normal PBMCs. These results indicate that AhR is also up-regulated and activated in some primary ATL cells. In the present study, we provided evidence that the elevated expression of AhR was attributable to the HTLV-1 transactivator, Tax. However, it is questionable whether Tax alone could explain the in vivo activation of AhR because the expression of Tax in primary ATL cells is known to be extremely low [7]. In addition, compared with HL60 cells, the Tax-induced expression levels of AhR-mRNA were very weak (Fig. 3). Therefore, it is likely that transactivation by Tax is not the only mechanism underlying AhR activation in primary ATL cells.

In the present study, we provided evidence of the up-regulation and activation of AhR in ATL, although the molecular mechanism and roles of AhR in ATL leukemogenesis are now under investigation. The Rel/NF-κB consists of a family of five proteins (p50, p52, RelA, RelB, and c-rel) which regulate a variety of physiological aspects of immune and inflammatory responses. These proteins bind to 10 bp DNA sites (κB sites) as a dimer and directly regulate gene transcription [25]. NF-κB is constitutively activated in primary ATL cells as a dimer of p50/p50 and p50/RelA [26], and thought to contribute to the deregulated proliferation of primary ATL cells. A direct interaction between AhR and NF-κB signaling pathways has been reported [27,28]. For instance, the RelA NF-κB subunit and the AhR were recently demonstrated to cooperate in transactivating the c-*myc* promoter in mammary cells [28]. Thus, it could be that the activated AhR influences overall NF-κB activation in primary ATL cells. AP-1 is a transcription factor complex composed of members of the Fos family and the Jun family. AP-1 has been shown to alter gene expression in response to growth factors, cytokines, tumor promoters, and carcinogens. It is believed that deregulation and/or imbalance in AP-1 complexes may contribute in part to the malignant transformation. In primary ATL cells, AP-1 complexes containing *Jun* D are highly expressed and thought to be involved in leukemogenesis, however, the precise mechanism of their up-regulation remains to be determined [29]. Interestingly, *Jun* D up-regulation has been shown to be attributable to the AhR-ARNT pathway [14]. Thus, it is likely that the AhR may also be involved in this type of leukemogenesis.

In conclusion, this work provided the first evidence for constitutive production and activation of AhR in both ATL cell lines and primary ATL cells. We further demonstrated the potential role of Tax in AhR-expression in ATL cells. A better understanding of the molecular mechanisms of AhR in ATL will shed more light on the leukemogenesis of ATL, and possibly open new avenues toward therapeutic strategies for this currently incurable leukemia.

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