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Differences in phosphorylation of the IL-2R associated JAK/STAT proteins between HTLV-I (+), IL-2-independent and IL-2-dependent cell lines and uncultured leukemic cells from patients with adult T-cell lymphoma/leukemia☆

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Abstract

To determine activation status of the IL-2R-associated (Jak/STAT) pathway in the HTLV-I infected cells, we examined tyrosine phosphorylation of Jak3, STAT3, and STAT5 in several HTLV-I (+) T-cell lines and in uncultured leukemic T cells isolated from patients with adult T-cell lymphoma/leukemia (ATLL). Constitutive basal phosphorylation of Jak3 and, usually, STAT3 and STAT5 was detected in all four IL-2-independent cell lines tested, but in none of the three IL-2-dependent cell lines. Similarly, there was no detectable basal phosphorylation of Jak3 and STAT5 in the leukemic cells from ATLL patients (0/8 and 0/3, respectively). However, stimulation with IL-2 resulted in Jak3 and STAT5 phosphorylation in both leukemic ATLL cells and IL-2-dependent lines. Furthermore, expression of SHP-l phosphatase which is a negative regulator of cytokine receptor signaling, was lost in most IL-2 independent cell lines (3/4) but not in the leukemic ATLL cells (0/3). Finally, the HTLV-I (+) T-cell lines (313) but not the control, HTLV-I (−) T-cell lines were resistant to rapamycin and its novel analog RAD. We conclude that (l) HTLV-I infection per se does not result in a constitutive phosphorylation of the Jak3, STAT3, and STAT5 proteins; (2) malignant transformation in at least some cases of ATLL does not require the constitutive, but may require IL-2-induced, activation of the IL-2R Jak/STAT pathway; and (3) there are major differences in T-cell immortalization mechanism(s) which appear to involve SHP-l and target molecules for rapamycin and RAD.

Keywords: IL-2R signaling; Malignant T cells; JAK5 kinase; STAT 5 protein; SHP-1 phosphatase

1. Introduction

IL-2 is a key cytokine involved in proliferation and differentiation of T lymphocytes and other cells of the immune system. IL-2 signaling involves dimerization of the β chain and common γ chain (γc) of the IL-2 receptor (IL-2R) [1]. In addition to IL-2R, the γc is a component of several other cytokine receptors; it can co-dimerize with cytokine-specific chains to transduce signals mediated by IL-4, IL-7, IL-9, and IL-15 [2–5]. Signaling by cytokine receptors involves sequential activation of the Janus-family tyrosine kineses (Jaks) and signal transducer and activator of transcription proteins (STATs) (reviewed in [6]). Binding of IL-2 to the IL-2R results in activation of Jakl and Jak3 kineses and tyrosine phosphorylation of several substrates, includ-

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ing Jakl and Jak3 themselves, as well as the IL-2R β and γc chains [7]. The phosphorylated IL-2R chains recruit proteins such as STAT5 [8,9] and, in phytohemagglutinin preactivated T-cell blasts, STAT3 [8,10]. The STATs, upon phosphorylation, presumably by the Jaks, translocate into the nucleus and bind to DNA to initiate transcription of the IL-2 responsive genes. Involvement of Jak3 is crucial for transduction of signals mediated by rc because mutations of Jak3 result in severe immunodeficiency in patients [11,12] and mice [13,14] which mimics the immunodeficiency seen in mutations of the γc itself [15–18]. The immunodeficiency in the Jak3-deficient mice can be reversed by transfection of the hematopoietic [19] or embryonic [20] cells with functional, wild-type Jak3. Stat 5 appears also critical for activation of normal, postthymic T cells, because mature T cells derived from mice deficient in both a and b isoforms of STAT5 failed to yield proliferative response upon stimulation [21].

Adult T-cell lymphoma leukemia (ATLL) is a malignancy affecting mature T lymphocytes. In most cases, the malignant ATLL cells display the CD3 +, CD4 +, CD8 −, CD7 −, and T-cell receptor (TCR) α/β(+) phenotype. Characteristically, they express activation antigens such as HLA-DR and CD25 (IL2Rα chain) [22]. Numerous epidemiological and clinical studies have established the association of HTLV-I with ATLL as well as other diseases including tropical spastic paraparesis/HTLV-I associated myelopathy (TSP/HAM) [23]. Depending on the clinical course, the extent of the disease and the serum calcium level, ATLL can be divided into four clinical subtypes: acute, chronic, lymphomatous, and smoldering [24]. In all these variants, patients have serum antibodies to HTLV-I and clonal integration of one or few copies of the virus in the DNA of the malignant cells [25,26]. Patients with detectable monoclonal or oligoclonal populations and elevated PBMC counts are at increased risk of developing an overt ATLL disease [27]. However, the low frequency of ATLL (4–5%) among HTLV-I infected individuals [28] and the long average time interval between the occurrence of infection and the development of malignancy (20–30 years) indicate that additional events are required for malignant transformation of T-cells.

Experiments with HTLV-I transformed T-cell lines suggested that the virus may induce basal constitutive activation of the IL-2R associated Jak/STAT pathway, and that this pathway may be involved in HTLV-I-mediated T-cell transformation [29,30]. However, we previously found that constitutive activation of IL-2R-associated Jak/STAT signaling pathway also occurs in HTLV-I (−) malignant cells from patients with cutaneous anaplastic large T-cell lymphoma (ALCL) [31]. This finding indicated that the constitutive activation of IL-2R Jak/STAT pathway in transformed T cells may not be due to to the HTLV-I infection. To explore further the putative role of HTLV-I infection in the activation of this pathway, we examined several HTLV-I (+) T-cell lines that differ in their IL-2 dependency for the basal and IL-2-induced phosphorylation of the Jak3, STAT3, and STAT5 proteins. Furthermore, we also examined uncultured leukemic cells isolated directly from patients with ATLL. Our data demonstrate that the constitutive phosphorylation of the IL-2R associated Jak and STAT proteins is detectable only in the IL-2 independent HTLV-I (+) cell lines. It is not seen in the IL-2 dependent HTLV-I (+) lines and the leukemic ATLL cells. Both these cell types, however, phosphorylate Jak3 and STAT5 in response to IL-2. Furthermore, most of the IL-2 independent cell lines, but none of the leukemic ATLL cells, lacked expression of SHP-1 which down-regulates phosphorylation of Jak3. Finally, the HTLV-I (+) lines, in contrast to the control, HTLV-I (−) T-cell lines showed resistance to the IL-2R-signaling inhibitors rapamycin and its novel analog RAD. Implications of these findings for pathogenesis of the HTLV-I infection and ATLL are discussed.

2. Materials and methods

2.1. Patients

A total of eight ATLL patients, two from the US and six from Japan, were tested. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll centrifugation [31,32] from two patients diagnosed at the University of Pennsylvania with ATLL based on clinical, histopathological, and immunophenotypic criteria. Both patients developed anti-HTLV-I antibodies as determined by Western Blot of serum proteins, peripheral white blood cell count greater than 50×10^3/ul with a predominant lymphocytosis (greater than 50%), abnormal pathognomonic cells with multi-lobulated, flower-like nuclei on peripheral blood smear, serum LDH greater than 1.5 times upper limit of normal, and corrected Ca^{2+} of greater than 14 mg/dl. The above findings fulfilled criteria for the acute form of ATLL [24]. Serum concentrations of soluble IL-2R were 59 520 U/ml (patient #1) and 45 740 U/ml (patient #2) (normal < 1000 U/ml) indicating high tumor burdens ([33–35]). Flow cytometry analysis revealed that greater than 95% of PBMC from these patients had phenotypes consistent with ATLL cells (CD3 +, CD4 +, CD7 −, CD25 +, HLA DR +). Fig. 1 shows the cells with characteristic flower-like nuclei and salient flow cytometry data from patient #1. Six Japanese patients were diagnosed with ATLL (five acute and one chronic form) at the Kagoshima University using the same criteria as described above. Ficoll-

isolated PBMC were cryopreserved in DMSO/FBS containing medium and thawed shortly before being used for experiments.

2.2. Cell lines

Two types of HTLV-I (+) cell lines were used: IL-2 independent cell lines derived mostly from ATLL patients and IL-2 dependent cell lines derived from nonleukemic, TSP/HAM patients. The HTLV-I (+), IL-2 independent cell lines were ATL-2: CD4+ CD8− T cells originally cultured in IL-2 from PBMC of a patient with acute ATLL [36]; C91PL: cord blood T cell line established by co-culturing cord blood cells with known ATLL cells in the presence of IL-2 [37]; C1OMJ2: established from HTLV-I infected lymphocytes in a patient with ATLL [38]; and HUT102B: constitutive producer of HTLV-I derived from the lymph node of a patient with HTLV-I, also initially dependent on IL-2 [39,40]. HTLV-I (+), IL-2 dependent cell lines, Boul, Laf, and Cor, were derived from HTLV-I (+) non-leukemic patients with TSP/HAM [41]. These cell lines required 50–100 U/ml IL-2 for optimal growth and did not become IL-2 independent even after multiple passages. For controls, five HTLV-I (−) cell lines were used. The Sez4 line, kindly provided by T. Abrams, Hahnemann University was derived from a patient with a leukemic phase of cutaneous T-cell lymphoma (Sezary Syndrome) and bears close morphological, phenotypic, and genotypic resemblance to the fresh tumor cells [42]. The Sez4 line requires IL-2 (50–100 U/ml) for continuous proliferation. The YT line [43], a human NK cell line, was kindly provided by J. Yodoi, Kyoto University, Kyoto, Japan. PB-1, 2A, and 2B T-cell lines which were established from a patient with a progressive cutaneous T-cell lymphoproliferative disorder have been described in detail previously [31,44,45]. The PB-1 cell line was obtained at a relatively early stage of the patient's cutaneous T-cell lymphoma from neoplastic T-cells circulating in peripheral blood. The 2A and 2B lines were established at a later, more aggressive stage from two separate skin nodules, which represented a high-grade, T-cell anaplastic large-cell lymphoma. All five control cell lines were determined to be HTLV-I (−) by PCR detection of the HTLV *tax* gene (Table 1). All the cell lines were propagated in a complete RPMI 1640 medium containing 10% FBS (Hyclone, Logan, UT), 1% L-glutamine (M.A. Bioproducts, Walkersville, MD), and 1% penicillin/streptomycin/fungizone mixture (M.A. Bioproducts).

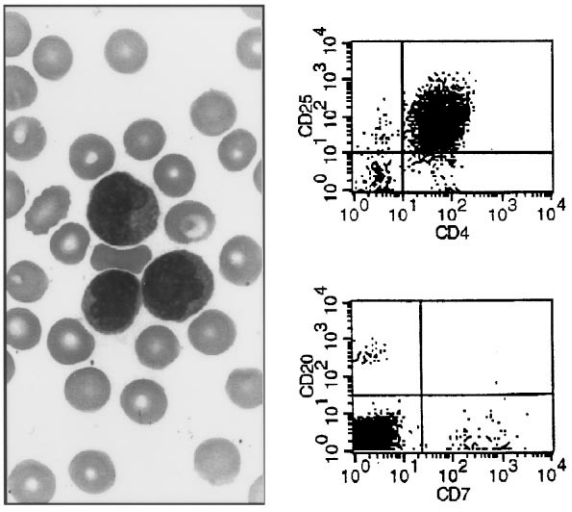

Fig. 1. Left panel: a representative peripheral blood smear from ATLL patient #1 showing pathognomonic large cells with flower-like nuclei (Wright–Giemsa, magnification 1000×). Right panel: flow cytometry analysis of the patients PBMC which shows that >95% cells exhibit a CD3+, CD4+, CD8−, CD7−, HLA-DR+ and CD25+ phenotype consistent with ATLL. Representative dot plots are shown where >95% of cells are CD4+/CD25+ and CD7−.

Table 1
HTLV-I status of the cell populations examined

Cell types	HTLV status	Method of detection
Control cell lines		
YT (NK-like)	−	PCR *tax*
Sez-4	−	PCR *tax*
PB-1	−	PCR *tax*
2A	−	PCR *tax*
2B	−	PCR *tax*
IL-2 dependent cell lines		
Boul	+	PCR *tax*
Laf	+	Immunofluorescence
Cor	+	Immunofluorescence
IL-2 independent cell lines		
ATL-2	+	PCR *tax*
C91PL	+	PCR *tax*
C10MJ2	+	PCR *tax*
HUT102	+	PCR *tax*
ATLL Patients (8)	+	Positive serology*

* Cells were also histopathologically and immunophenotypically consistent with HTLV-I infected cells.

2.3. Flow cytometry

Flow cytometry immunophenotyping of PBMC was performed using a standard panel of T- and B-cell reactive mAbs including the ones which recognize activation antigens HLA-DR and CD25 (Becton-Dickinson).

2.4. IL-2, antibodies (Ab), and immunosuppressive agents

Recombinant human IL-2 was kindly provided by C. Reynolds, NCI, Frederick, MD. Rabbit polyclonal Abs against JAK3, STAT3, STAT5, and SHP-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine 4G10 murine monoclonal Ab was purchased from UBI (Lake Placid, NY). Peroxidase-conjugated donkey anti-mouse and goat anti-rabbit Abs were obtained from Jackson Jackson Immuno Research (West Grove, PA). Rapamycin and SZS RAD were kindly provided by, respectively, Wyeth-Ayerst (Princeton, NJ) and Novartis Pharma (Basel, Switzerland).

2.5. PCR/Southern blot analysis

A total of 1.0 ug of genomic DNA was added to 50 ml of standard buffer containing 1.5 mM $MgC1_2$, 1.25 mM dNTP mix, 15 pmol of 3′ and 5′ primers, and 2.5 U Taq polymerase (Perkin-Elmer, Norwalk, CT). Reaction mixtures were amplified for 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Oligonucleotide primers for conserved sequences of the HTLV-I/II *tax* gene, SK43 and SK44 [46], synthesized by Research Genetics, Huntsville, AL, were used for amplification of HTLV-I/II *tax* gene sequences. Amplification products were separated on 2% agarose gels, blotted and probed with ^{32}P-labeled SK45 probe [46] from Research Genetics as described before [47]. DNA from HTLV-II infected cell line (MoT) served as positive control.

2.6. Protein expression and phosphorylation

These assays were performed as described [31,48]. In brief, the cells ($l0 \times 10^6$) were washed, exposed for 5 min to medium or 500 U IL-2, lysed for 20 min in l ml ice-cold lysis buffer (0.5% NP-40, 10 mM Tris–HC1 (pH 7.4), 150 mM NaCl, 0.4 mM EDTA, 1 mM sodium orthovanadate, 0.5 mM PMSF, 10 mM NaF, and 3 ug/ml each of pepstatin, leupeptin, chymostatin, and aprotinin: Sigma). The lysates were centrifuged at 15 000 rpm for 10 min. Next, the supernatants were precleared overnight at 4°C with protein A-sepharose (Sigma, St Louis, MO), incubated with the anti-Jak3, -STAT3, -STAT5, or SHP-1 Ab, and protein A-sepharose, washed, boiled, suspended in reducing SDS loading buffer, separated on a 10% polyacrylamide-SDS gel, and transferred electrophoretically to hybridization transfer membranes. The membranes were blocked with 2% bovine serum albumin in TBST buffer (l0 mM Tris–HCl (pH 7.4), 75 mM NaCl, l mM EDTA, 0.1% Tween 20) for at least 2 h at room temperature or overnight in a cold room. To detect protein phosphorylation, the membranes were incubated with 4G10 Ab, washed, incubated with donkey anti-mouse, peroxidase-conjugated Ab and washed again. To detect protein expression the membranes were incubated with the same Jak3, -STAT3, -STAT5, or -SHP-1 Abs which were used for precipitation. Blots were developed using the ECL chemiluminescence reagents (Amersham Life Science, Arlington Heights, IL).

2.7. Proliferation assays

These tests were performed as described previously [31,32]. In brief, the cell lines or PHA-stimulated PBMC were cultured for either 10 or 18 h in triplicate at 2×10^4 cells/well in the presence of various concentrations of the immunosupressive drugs; rapamycin or RAD. After 14 h pulse with 0.5 μCi of [^{3}H]thymidine, radioactivity of the cells was measured.

3. Results

3.1. Determination of the HTLV-I infection status

The HTLV-I (+) status of all cell populations used was determined by detection of viral *tax* gene in genomic DNA, detection of viral gene products via immunofluorescence, or in patients, by clinical, histopathologic and immunophenotypic criteria for ATLL in combination with serological evidence for HTLV-I infection (see Section 2.1 and Fig. 1). Table 1 summarizes the HTLV-I status of the cell populations examined. Controls used included cutaneous T-cell lymphoma cell lines: Sez4 (IL-2 dependent) and PB-1, 2A, and 2B (all IL-2 independent) and an NK cell line, YT. All control cell lines were shown to be HTLV-I (−) by the PCR assay for the HTLV-I *tax* gene.

3.2. Phosphorylation of IL-2R associated Jak/STAT proteins in IL-2 independent T-cell lines

The few HTLV-I (+), IL-2 independent T-cell lines tested to date have all been reported to display constitutive activation of the IL-2R associated Jak/STAT pathway [26,27]. We explored the extent of these findings by analyzing four HTLV-I (+), IL-2 independent T-cell lines (ATL-2, C1OMJ2, C91PL, HUT 102), two of which (ATL-2, C1OMJ2) have not been examined to date. Fig. 2A and Table 2 show that all the cell lines demonstrate a strong basal, constitutive phosphorylation of Jak3 with only the HUT102 cell line showing a slight augmentation in response to IL-2. STAT3 and STAT5 are also strongly, constitutively phosphorylated in the ATL-2 and C10MJ2 cell lines (Fig. 2B, C, and Table 2). Interestingly, constitutive phosphorylation of

the entire Jak3/STAT3/STAT5 pathway does not seem to be a universal feature even in these cell lines because two of the lines (C91PL and HUT102B) did not exhibit any detectable, basal phosphorylation of STAT5 in repeated experiments although they expressed the protein and strongly phosphorylated STAT5 in response to IL-2 (Fig. 2C, Table 2). This finding implies a dissociation between Jak3 and STAT5 phosphorylation and suggests that the signals transduced by Jak3 may not necessarily always depend on phosphorylation of STAT5. In addition, one cell line, C91PL, showed a lack of STAT3 phosphorylation in the presence or absence of IL-2. This pattern of response is similar to that seen in resting PBMC rather than mitogen pre-activated T-cells [8,26,28]. The control Sez4 malignant T-cell line which is HTLV-I (−) and, noteworthy, IL-2

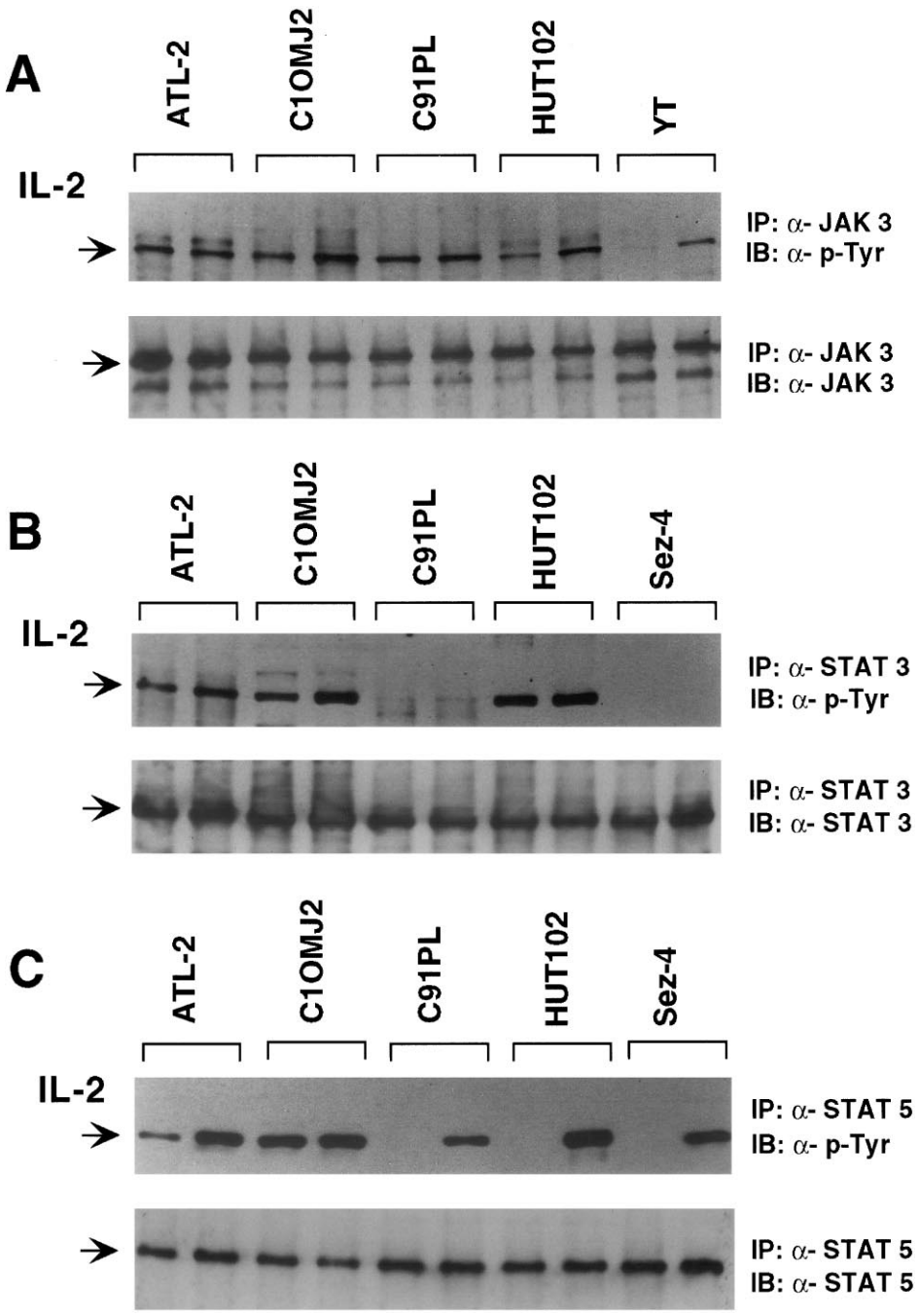

Fig. 2. Phosphorylation of proteins associated with IL-2R signal transduction pathway in HTLV-I positive, IL-2 independent cell lines (ATL-2, C9lPL, C1OMJ2, HUT102B) derived from leukemic patients without (−) and with (+) stimulation by IL-2: (A) Jak3, (B) STAT3, and (C) STAT5. The cell lysates were immunoprecipitated with the anti-Jak3, -STAT3, and -STAT5 Ab, electrophoretically separated, transferred to a membrane and probed with an Ab (4G10) which recognizes phosphorylated but not non-phosphorylated tyrosines. Loading of equal sample volumes was confirmed by the subsesquent immunoblotting with the Ab used for immunoprecipitation after removal of the bound 4G10 Ab.

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