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Foxp3-dependent Transformation of Human Primary CD4+ T Lymphocytes by the Retroviral Protein Tax

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Abstract

The retroviral Tax proteins of human T cell leukemia virus type 1 and 2 (HTLV-1 and -2) are highly homologous viral transactivators. Both viral proteins can immortalize human primary CD4+ memory T cells, but when expressed alone they rarely transform T cells. In the present study, we found that the Tax proteins displayed a differential ability to immortalize human CD4+Foxp3+ T cells with characteristic expression of CTLA-4 and GITR. Because epidermal growth factor receptor (EGFR) was reportedly expressed and activated in a subset of CD4+Foxp3+ T cells, we introduced an activated EGFR into Tax-immortalized CD4+Foxp3+ T cells. We observed that these modified cells were grown independently of exogenous IL-2, correlating with a T cell transformation phenotype. In Tax-immortalized CD4+Foxp3- T cells, ectopic expression of Foxp3 was a prerequisite for Tax transformation of T cells. Accordingly, treatment of the transformed T cells with erlotinib, a selective inhibitor of EGFR, induced degradation of EGFR in lysosome, consequently causing T cell growth inhibition. Further, we identified autophagy as a crucial cellular survival pathway for the transformed T cells. Silencing key autophagy molecules including Beclin1, Atg5 and PI3 kinase class III (PI3KC3) resulted in drastic impairment of T cell growth. Our data, therefore, unveiled a previously unidentified role of

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Conflict of interest

The authors declare no conflict of interest.

Authors' contributions

LC and DL designed, performed experiments and analyzed the data. LC wrote the manuscript. YZ and HZ conducted parts of the experiments. HC analyzed the data and modified the manuscript.

Foxp3 in T cell transformation, providing a molecular basis for HTLV-1 transformation of CD4⁺Foxp3⁺ T cells.

Keywords

HTLV Tax; Foxp3; Stat3; NF- κ B; transformation; autophagy

Introduction

Human T cell leukemia virus type 1 and type 2 (HTLV-1 and HTLV-2 respectively) are two related human retroviruses of bloodborne pathogens. HTLV-1 is the etiological factor that causes adult T cell leukemia and lymphoma (ATL) in 20 million of HTLV-1-infected patients [1,2]. Although HTLV-2 is detected in CD8 T cells from a patient with hairy cell leukemia, a rare type of leukemia that affects B cells [3], a causative link between HTLV-2 infection and hairy cell leukemia has not been established. HTLV-1 viral genome is known to encode two oncogenic products, Tax-1 and HBZ, an antisense gene product that is constitutively transcribed in ATL cells [4,5]. Tax-1 is the first recognized viral component that displays oncogenic ability in human primary CD4⁺ T lymphocytes, humanized mouse model and transgenic mice [4,6]. Increasing evidence also supports the important role of HBZ during leukemogenesis of HTLV-1 [7,8].

The underlying mechanism of HTLV-1-mediated T cell transformation remains incompletely understood. Neither Tax nor HBZ is able to transform human primary lymphocytes. It is well recognized that expression of Tax is essential for initiation of HTLV-1-associated oncogenesis [4,6]. This concept is based on several experimental findings. The molecular clone of HTLV-1 with the disrupted *tax* gene has no capacity to transform human T cells [9,10]. In addition, Tax, not HBZ, induces immortalization of human CD4⁺ memory T cells, a crucial step leading to T cell malignancy [11]. Further, the ability of Tax in imitating T cell activation signaling, in promoting cell cycle progression and in causing genomic damage correlates with its role in oncogenic initiation [4,12,13]. Although it is not clear if HBZ is required during the early stage of oncogenesis, this viral protein was found constitutively expressed at the leukemia stage, and silencing HBZ led to growth inhibition of the leukemia cells [7,14]. A consensus view for HTLV-1-mediated T cell transformation is that Tax-1 by itself is inadequate to fully transform mature CD4⁺ T cells and it requires cooperation with HBZ to overcome Tax-1-induced cell senescence, thereby promoting oncogenesis [15].

HTLV-1-transformed T cells demonstrate a CD4⁺ Foxp3⁺ immunophenotype [16]. HBZ, not Tax, is able to upregulate Foxp3 expression [17]. Foxp3 is a master regulator of immunity in lymphoid tissues and a specific marker of regulatory T cells (Treg) [18–20]. The Treg cells function as a potent immune suppressor that restrains the activity of self-reactive cytotoxic T cells and inhibits proliferation of effector T cells. Loss of Treg cells is associated with autoimmune disease and conversely, hyper-activation of Treg cells may facilitate tumor growth and metastasis, probably by suppressing anti-tumor immunity [21,22]. The Foxp3⁺ tumors imitate Treg's immune suppressive function, which is

associated with poor prognosis in certain types of cancer [23,24]. In HTLV-1-associated ATL, the immunodeficiency phenotype manifests at certain stage of leukemia development [25]. However, in HTLV-1-associated neurological diseases, the presence of hyper reactive T cells to viral antigens suggested that HTLV-1-infected Foxp3 cells could be converted into Th1-like cells [26]. Yet, it is still not clear about the role of Foxp3 in HTLV-1-mediated transformation of T cells. In the present study, we report our new finding that Foxp3 is a prerequisite for Tax transformation of human differentiated CD4⁺ T cells. In addition, we have found that autophagy molecules play a key role in promoting survival and proliferation of Tax-transformed T cells.

Materials and Methods

Cell cultures and antibodies

TX2-1, TX2-2, TX2-3, TX2-4, PTX4-1 and PL9-1 cell lines were described previously [27,28], and were cultured in RPMI1640 medium containing 10% fetal bovine serum and 100 units/ml of recombinant IL-2. MT-2, MT-4 and Jurkat cells were obtained from AIDS research and reference reagent program (NIAID, National Institutes of Health). SLB-1 and MT-1 cell lines were described previously (28). Primary human CD4⁺ and CD8⁺ T cells were isolated from healthy blood donors and were enriched using anti-CD4 and anti-CD8 magnetic beads (Invitrogen).

Antibodies for Foxp3, EGFR and GFP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Tyr(PY99) antibody was from BD Transduction Lab (San Diego, CA). DMSO, MG-132, chloroquine and erlotinib were purchased from Sigma (St Louis, MO).

Lentivirus vectors and lentivirus production

Lentivirus constructs for Tax-GFP and the CD3-EGFR chimera were described previously [27,35]. Lentivirus vectors for Beclin1- and Atg5-specific shRNAs were purchased from Open Biosystems (Pittsburgh, PA). Lentivirus vector for PI3KC3-specific shRNA was purchased from Thermo Scientific (Grand Island, NY, USA). The Foxp3 cDNA was PCR-cloned, constructed in the lentivirus vector and sequence-verified. The procedure for lentiviral production and concentration was described previously [27].

Immunophenotyping analysis by FACS

The immunophenotypes of the Tax-immortalized and transformed T cell lines were stained with APC-conjugated antibodies for surface molecules and intracellular proteins including CTLA-4, GITR, CD45, CD39, CD73, HLA-DR and Foxp3 (eBioscience), according to the manufacturer's instructions. The stained cells were analyzed with FACS.

Cell proliferation assay and electrophoretic gel mobility shift assay (EMSA)

Cell proliferation assay was performed using tetrazolium compound-based CellTiter 96® Aqueous One Solution cell proliferation (MTS) assay (Promega, Madison, WI). Cell viability was measured by trypan blue exclusion assay. Cells were collected and re-suspended in fresh complete medium. An aliquot of cells was added to an equal volume of

trypan blue. The viable cells were measured by trypan blue exclusion assay. Each experiment was conducted in triplicate and the results were indicated as the mean $\pm$ SD. The sequences of the probes for NF- κ B, Stat3, AP-1 and OCT1 and EMSA were previously reported [27].

Results

The Tax proteins differentially immortalize human CD4⁺Foxp3⁺ T cells

We previously reported establishment of Tax-immortalized, human primary CD4⁺ T cells from healthy blood donors, showing the cytokine expression patterns that represented distinct subsets of helper T cells [27,28]. Although all of these established T cell lines were CD4⁺CD25⁺, a characteristic immunophenotype of human regulatory T cells (Treg), the nature of these T cell subsets remained to be elusive [27,28]. To explore the identity of these T cell subsets, we first examined the expression status of Foxp3. As shown in Fig. 1A and 1B, three out of four HTLV-2 (Tax-2)-established T cell lines expressed Foxp3. In contrast, HTLV-1 Tax (Tax-1)-immortalized T cell lines including PTX4-1 and PL9-1 and several HTLV-1-transformed T cell lines including SLB-1, MT-2, MT-4 and MT-1 were Foxp3-negative (Fig. 1A). Foxp3 was not detected in primary CD4 and CD8 T cells from healthy donors (Fig. 1A), because Foxp3⁺ cells constitute less than 5% of total lymphocytes. Further analysis demonstrated that these Foxp3⁺ T cell lines displayed a CTLA-4⁺GITR⁺CD39⁺HLA-DR⁺ immunophenotype (Fig. 1C), which was consistent with the phenotype of activated Treg cells [29,30]. As previously shown that these Foxp3⁺ T cells expressed variable amounts of Treg-related cytokines including IL-10 and TGF β [27], we concluded that Tax-2 selectively immortalized human CD4⁺Foxp3⁺ T cells.

These findings were somewhat unexpected, since it has been documented that HTLV-2 preferentially infects CD8⁺ T cells, causing lymphocytosis but not leukemia [31]. Besides, natural Treg cells (nTreg) with the CD4⁺CD25⁺Foxp3⁺ immunophenotype are very rare in peripheral blood [32]. Furthermore, HTLV-1-associated ATL cells are CD4⁺CD25⁺Foxp3⁺ T cells in more than 80% of clinical ATL cases [33], suggesting that HTLV-1 virus, but not Tax-1, selects the subsets of CD4⁺ T cells for infection and malignant transformation in patients. The observation that the *in vitro* cultured, HTLV-1-transformed cells were Foxp3 negative was consistent with the previous report that Foxp3 was only transiently upregulated during HTLV-1 leukemogenesis [34].

Foxp3 is a prerequisite for Tax induction of T cell transformation

Foxp3 was obviously not required for Tax immortalization of human primary T cells, however it might be important for Tax induction of T cell transformation. It is known that Tax alone is necessary but not sufficient to fully transform primary human T cells, and an oncogenic cooperation would be required during leukemogenic process. To test this notion, we first selected two Tax-2-immortalized T cell lines, TX2-1 (Foxp3⁻) and TX2-4 (Foxp3⁺), based on their comparable growth rate. We introduced a variety of cellular and viral oncogenes, such as activated EGFR (CD3-EGFR) [35], v-Src, H-RasV12 and c-Myc, into Tax-immortalized T cells as depicted in Fig. 2A. We found that only ectopic expression of the activated EGFR supported IL-2-independent growth of the Tax-immortalized

CD4⁺Foxp3⁺ T cells (Fig. 2A, 2B and 2C). It has been well recognized that the cytokine-independent growth of human primary T lymphocytes is a T cell transformation phenotype [4].

Next, to determine the role of Foxp3 in Tax-mediated transformation of T cells, we ectopically expressed Foxp3 in the Foxp3-negative TX2-1 cells. The Foxp3-expressing TX2-1 cells remained not transformed since their growth was still exclusively dependent on the exogenous IL-2 (Fig. 2D, left panel). However, when the activated EGFR was introduced into the Foxp3-expressing TX2-1 cells, these cells became transformed (Fig. 2D, right panel). Similarly, PTX4-1 cells, the Tax-1-immortalized T cells, were Foxp3 negative (Fig. 1A). We ectopically expressed Foxp3 in PTX4-1 cells to generate the Foxp3-expressing PTX4-1f cells (Fig. 2E). We found that the growth of PTX4-1f cells remained to be dependent on recombinant IL-2 (data not shown). Introduction of the activated EGFR to generate PTX4-1f-E cells led to IL-2-independent proliferation of these cells (data not shown). We noticed that the levels of Tax1-GFP in PTX4-1f and PTX4-1f-E cells were significantly higher than that in PTX4-1 cells. This phenomenon could be caused by selective variation in which Foxp3 selected the subsets of PTX4-1 cells that expressed a higher level of Tax1-GFP to allow them to grow optimally. The endogenous EGFR was not detected in Tax-transformed T cells, however EGFR was reportedly expressed and activated in a subset of human Treg cells [36]. Further, the activity of NF- κ B or Stat3 were not altered significantly in Tax-immortalized or transformed T cells (Fig. 2F). Clearly, we showed here that Foxp3 was a prerequisite for Tax transformation of human primary T cells and its transformation-promoting activity appeared not to be related to the alteration of the NF- κ B or Stat3 activity.

We further analyzed the immunophenotype of Tax-transformed TX2-1 and TX2-4 cells. The majority of the surface receptors such as CTLA-4 and GITR remained unchanged, however the expressions of CD3 and TCR $\alpha\beta$ were noticeably downregulated (Fig. 3), in comparison with the Tax-immortalized T cells [27]. This finding was consistent with the feature that HTLV-1-transformed T cells and leukemia cells displayed downregulated CD3 and TCR $\alpha\beta$ [37]. Together, our data supported the theory that oncogenic transformation of human cells requires cooperation of at least two oncogenes.

Expression of the activated EGFR and autophagy molecules are required for maintaining Tax transformation of Foxp3⁺ T cells

We examined a potential role of EGFR in maintenance of T cell transformation phenotype by treating the transformed T cells with erlotinib, a selective inhibitor of the activated EGFR [38]. We found that this drug induced drastic reduction of the activated EGFR (Fig. 4A). To determine if the activated EGFR is degraded in the proteasome or in the lysosome, we pre-treated the cells with MG-132, a proteasome inhibitor, or with chloroquine, a compound that blocks fusion of autophagosome with lysosome, followed by adding erlotinib as indicated doses. We observed that chloroquine, but not MG-132, was capable of rescuing erlotinib-induced degradation of the activated EGFR (Fig. 4A, middle and bottom panels). Expectedly, the erlotinib treatment reduced cell viability of the Tax-transformed, Foxp3⁺ T cells in a dose-dependent manner (Fig. 4B and 4C). This finding supported a critical role of

the activated EGFR in maintaining the transformation phenotype of Tax-transformed T cells.

Dysregulation of autophagy has been implicated in playing a crucial role in oncogenesis and cancer metastasis [39]. We previously showed that the Tax protein increased autophagic flux by regulating the activity of I κ B kinases, and this process appeared to be crucial for survival of HTLV-1-transformed T cells. To investigate the role of autophagy in maintaining the transformation phenotype of Tax-transformed, Foxp3⁺ T cells, we silenced key autophagy molecules including Beclin1, Atg5 and PI3 kinase class III (PI3KC3). We found that silencing either one of these autophagy molecules led to drastic reduction of cell viability and proliferation in both Tax-transformed, Foxp3⁺ T cell lines, TX2-4E and TX2-1fE (Fig. 4D and 4E). The knockdown of the autophagy molecules in TX2-1fE cells was more efficient than that in TX2-4E cells, correlating with a more potent suppression of the growth of TX2-1fE cells. Our data, therefore, validated a crucial role of autophagy molecules in the maintenance of T cell transformation phenotype in the context of Tax transformation of Foxp3⁺ T cells.

Discussion

We have identified a new role of Foxp3 in assisting the retroviral Tax-induced transformation of human primary CD4⁺ T lymphocytes. Although the molecule mechanism of Foxp3 in assisting Tax-mediated transformation of human T cells remains unclear, our study unveiled a new role of this master regulator in T cell malignancy. The Foxp3 expression is not necessary for Tax immortalization of CD4⁺ T cells but plays a key role in Tax-mediated T cell transformation. We have observed that although Tax-immortalized PTX4-1 cells are proliferating in culture, they frequently experience spontaneous cell death. This phenomenon is consistent with the Tax's cytotoxicity and induction of cell senescence in subsets of T cells. Such cytotoxicity is reduced by ectopic expression of Foxp3 in PTX4-1 cells, though the growth of these cells remains to be dependent on exogenous IL-2. Thus, it appears that Foxp3 may have a crucial role in overcoming Tax's cytotoxicity, allowing HTLV-1-transformed T cells to proliferate. In HTLV-1-infected T cells, HBZ induces Foxp3 expression and unlocks Tax-1-mediated senescence [15,17]. Therefore, our finding supports the theory that the oncogenic cooperation between Tax and HBZ induces malignant transformation of human CD4⁺ T cells. It is highly desirable to test this theory by co-expressing Tax and HBZ in human differentiated T cells to determine their transforming ability. Furthermore, the observation that the Foxp3-expressing PTX4-1 cells are transformed by expression of the activated EGFR validates the theory that an oncogenic cooperation of at least two oncogenes is necessary to fully transform human cells.

Our data have also demonstrated a critical role of autophagy in the process of T cell transformation by Tax. Autophagy serves as a double-edged sword, which has been reported to play roles in either promoting cancer growth or suppressing tumor formation depending on the context and stage of cancer [39]. Increased autophagic flux has been shown to be crucial in other oncogenic virus-mediated cellular transformation [40]. Consistent with the cytoprotective role of autophagy, silencing key autophagy molecules such as Beclin1, Atg5 and PI3KC3 led to the impaired survival and growth in the context of Tax induction of T

cell transformation. Further studies are necessary to determine the detailed underlying mechanism of Foxp3 in HTLV-1 oncogenesis and to develop therapeutic molecules targeting the survival pathways during the process of leukemogenesis.

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Figure 1. The retroviral Tax proteins differentially immortalize human CD4+Foxp3+ T cells
 (A) The expression status of Foxp3 in Tax-immortalized T cell lines and HTLV-1-transformed T cell lines as determined by anti-FoxP3 immunoblot. (B) Intracellular staining using APC-conjugated anti-FoxP3 antibody. (C) Immunophenotypes of Tax-2-immortalized T cell lines, TX2-1, TX2-2, TX2-3 and TX2-4, as measured by FACS. (D) Intracellular expression of CTLA-4 in TX2-2 and TX2-3 cell lines as detected by the intracellular staining method with APC-conjugated anti-CTLA-4 antibody.

Figure 2. Foxp3 is a prerequisite for Tax transformation of human CD4+ T cells by Tax
 (A) The diagram indicated that in Tax-immortalized TX2-4 cells (CD4+Foxp3+), introduction of CD3-EGFR led to their IL-2-independent growth. In contrast, expression of CD3-EGFR in TX2-1 cells (CD4+Foxp3-) was not sufficient to support IL-2-independent growth. Forced expression of Foxp3 in TX2-1 cells and subsequent introduction of CD3-EGFR led to their IL-2-independent proliferation. (B) Immunoblot analysis to verify the expressions of Foxp3, CD3-EGFR, phosphorylated CD3-EGFR and Tax-GFP. TX2-1f-C, TX2-1 cells transduced with Foxp3 lentivirus followed by expression of CD3zeta (control); TX2-1f-E, TX2-1 cells transduced with Foxp3 lentivirus followed by expression of CD3-EGFR; TX2-4C, TX2-4 cells transduced with CD3zeta (control) and TX2-4E, TX2-4 cells transduced with CD3-EGFR. (C) Cell viability of TX2-4C and TX2-4E cells in the presence or absence of recombinant IL-2 (100u/ml). (D) Cell viability of TX2-1f-C and TX2-2f-E cells in the presence or absence of recombinant IL-2 (100u/ml). (E) Immunoblot analysis of

Tax-1-immortalized and -transformed T cell lines using antibodies as indicated in figure. PTX4-1, Tax-1-immortalized T cell line; PTX4-1f, Foxp3-expressing PTX4-1 cells; PTX4-1f-E, PTX4-1f cells expressing CD3-EGFR. (F) The activities of NF- κ B and Stat3 in Tax-immortalized and -transformed T cell lines were analyzed with EMSA. SLB-1, an HTLV-1-transformed T cell line; PBLs, primary peripheral blood lymphocytes.

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Figure 3. Surface receptor expressions of Tax-transformed T cell lines TX2-4E and TX2-1f-E
 Expressions of cell surface molecules in Tax-2-transformed T cell lines TX2-4E and TX2-1f-E were analyzed with FACS using APC-conjugated antibodies indicated in the figures.

Figure 4.

Expression of the activated EGFR and autophagy molecules is required for maintaining Tax-transformation of CD4+Foxp3+ T cells. (A) TX2-4E and TX2-1f-E cells were pretreated with DMSO (controls), 5mM of MG-132 or 50mM of chloroquine for 1 hour. These cells were treated with DMSO or erlotinib at 6.25, 12.5 or 25 mM. 7 hours following the treatment, the whole cell lysates were examined with immunoblot. (B) and (C) TX2-4E and TX2-1f-E cells were treated with erlotinib at indicated doses for 24, 48 or 72 hours. Cell viability was determined with MTT assay. Silencing Beclin1, Atg5 and PI3KC3 using lentiviral transduction of specific shRNAs was performed in TX2-4E (D) or TX2-1f-E (E) cells. The cell viability was determined by trypan blue exclusion assay.