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HTLV-1 Tax and Adult T-cell Leukemia

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1. ABSTRACT

Human T-lymphotropic virus type I (HTLV-1) is the etiological agent of adult T-cell leukemia/lymphoma (ATL) and HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1 viral transactivator/oncoprotein, Tax, activates viral transcription and usurps regulatory mechanisms that are critical for cell growth and division to facilitate viral replication. The effects that Tax exerts on cells include potent NF-κB activation, cell cycle perturbation and cell transformation. How Tax influences ATL development is incompletely understood at present. While Tax-expression is needed at the early stages of cellular transformation, at later times most ATL cells do not express *tax*; therefore, genetic and epigenetic changes in HTLV-1-infected cells are believed to play an important role in the etiology of ATL. This review attempts to integrate recent literature on the biological activities of Tax and the properties of HTLV-1 transformed T-cells and ATL cells, and speculate on what cellular changes may collaborate with Tax to effect cell transformation and ATL development.

2. INTRODUCTION

Human T-cell lymphotropic virus type-1 (HTLV-1) was isolated in 1980 from a T cell line, Hut102, established from a patient initially diagnosed with cutaneous T-cell lymphoma (1, 2). Shortly after its discovery, HTLV-1 became etiologically linked to adult T-cell leukemia/lymphoma (ATL), a malignancy first described in 1977 in Japan (3, 4). Epidemiological studies indicate that HTLV-1 infection is endemic in parts of the Caribbean, the southern islands of Japan, parts of Africa, South America, and the Pacific islands of Melanesia and Papua New Guinea (5, 6). To date, HTLV-1 remains the only retrovirus associated with human malignancy. Unlike many other viruses, cell-free infection by HTLV-1 is highly inefficient, and most viral infection occurs via cell-cell contact. Tropism of HTLV-1 is largely for T-cells with ability of the virus to infect both CD4+ and CD8+ cells. In vivo, the virus is transmitted predominantly via breast milk, via transfusion of blood products containing HTLV-1-infected cells, and by sex. Interestingly, while HTLV-1 preferentially transforms CD4+ T-cells; the related HTLV-

2 virus has been shown to primarily induce proliferation of CD8+ T cells. The diseases caused by HTLV-1: adult T-cell leukemia (ATL), HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), HTLV-1 uveitis, HTLV-1 associated rheumatoid arthritis (7), and other inflammatory skin diseases have their etiologies in the dysregulated proliferation of T-cells and/or ensuing immune dysfunctions. Presently, there is no effective treatment for HTLV-1 infection and associated diseases. How HTLV-1 infection leads to the development of specific diseases also remain incompletely understood.

3. HTLV-1 RELATED DISEASES

3.1. Adult T-cell leukemia/lymphoma (ATL)

ATL is a rare T-cell malignancy characterized by hypercalcemia, hepatomegaly, splenomegaly, lymphadenopathy, skin involvement and presence of abnormal lymphocytes (3, 4). HTLV-1 causes ATL in a small percentage (2-6%) of infected individuals after a long latency period of up to 20-40 years. ATL patients show evidence of a monoclonal integration of HTLV-1 proviral DNA in proliferating $CD4^+CD25^+$ leukemia cells. These ATL cells emerge from a pool of HTLV-1-positive CD4+ T-cells that persist for decades in the infected individual through oligoclonal expansion. The genetic or epigenetic events that trigger full-blown malignancy are currently unknown. There are four ATL subtypes: acute, lymphomatous, chronic, and smoldering. The first two subtypes are associated with a rapidly progressing clinical course with a median survival time of 5-6 months. Smoldering and chronic ATL have a more indolent course and may represent transitional states towards acute ATL. ATL usually occurs at middle to old age among individuals who are infected in early childhood.

3.2. HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), HTLV-1 uveitis, and inflammatory skin diseases

HAM/TSP is a chronic neurological disorder caused by an inflammatory condition that results in demyelination of the spinal cord. It is characterized by progressive weakness and hyperreflexia of the lower limbs, bladder dysfunction, and impotence. The T lymphocytes from HAM/TSP patients often undergo spontaneous proliferation when cultured *in vitro*. Importantly, compared to asymptomatic HTLV-1 carriers, HAM/TSP patients have significantly higher proviral loads, suggesting that active HTLV-1 viral replication plays a key role in the development of the disease. A recent study has shown that a high level of Tax expression and low CD8+ anti-viral efficiency are correlated with high proviral load and HAM/TSP development (8). Unlike ATL, the onset of HAM/TSP may be rapid. HTLV-1 is also associated with uveitis, an inflammation of the iris. Finally, almost all HTLV-1 associated diseases except HTLV-1 uveitis, have skin manifestations preceding or concurring with disease onset. New data from a mouse model provides support that the observed dermatological pathology is due to an expansion of activated CD4+ T lymphocytes, driven by Tax-mediated activation of NF-κB, that traffic to the skin (9).

4. HTLV-1 PATHOGENESIS AND VIRAL TRANSACTIVATOR, TAX

The long incubation period and the low frequency of clinical progression to ATL suggest that complex viral and cellular events are involved in HTLV-1 pathogenesis. Indeed, statistical analysis has indicated that at least five independent genetic changes need to occur after HTLV-1 infection of T cells *in vivo* before development of ATL ensues (10). The molecular events that cause HTLV-1 infection to progress from clinical latency to T-cell malignancy and HAM/TSP is not clear but involves the critical viral transactivator/oncoprotein, Tax, which is capable of activating viral transcription and usurping regulatory mechanisms critical for cell growth and division to facilitate viral replication.

How Tax influences ATL development is incompletely understood at present. Many lines of evidence support the importance of Tax in the leukemogenic process: (a) HTLV-1 proviral DNAs in a significant fraction of ATL cells contain deletions. In these cells, however, the coding sequence of *tax* is preferentially retained, implicating a role of *tax* in ATL development (5, 6, 11). This notwithstanding, most ATL cells do not express HTLV-1 transcripts, suggesting that *tax* most likely affects the early stage of the disease process and its persistent expression is not needed for maintenance of the neoplasm (12). This property of *tax* sets it apart from other viral oncogenes such as the human papilloma virus E6 and E7 whose constitutive expression is needed for cell transformation. (b) Transgenic mice expressing *tax* (driven by the HTLV-1 LTR) developed neurofibroma, a tumor of mesenchymal tissue (13). Interestingly, one group of LTR-*tax* transgenic mice developed thymic atrophy and died soon after birth, consistent with the notion that Tax is cytotoxic (13). (c) Large granular lymphocytic leukemia has been found in mice transgenic for *tax* that is expressed from the T-cell specific granzyme B promoter (14). Leukemia of CD4+ T-cells, however, has not been observed in any of the *tax*-transgenic systems. The basis for the differences between the transgenic models and HTLV-1 pathogenesis in humans remains unclear. (d) *Tax* can transform Rat-1 fibroblast cells in culture. (e) Tax can immortalize primary human T cells causing the cells to proliferate indefinitely (15); although currently complete transformation of primary human cells by Tax-alone has not been achieved. (f) Tax exerts pleiotropic effects on cellular signal transduction pathways and cell cycle controls.

5. MECHANISMS OF TAX ACTION AND ATL DEVELOPMENT

5.1. Activation of HTLV-1 viral transcription by Tax

The mechanism by which Tax activates viral transcription is well understood. The viral transcriptional enhancer consists of three imperfect 21 bp repeats, each containing a cAMP response element (CRE) core flanked by 5' G-rich and 3' C-rich sequences. In the presence of Tax, gene expression driven by multiple copies of the 21-bp repeat element can increase up to 100-fold or higher. Cellular basic domain-leucine zipper (bZip) transcription

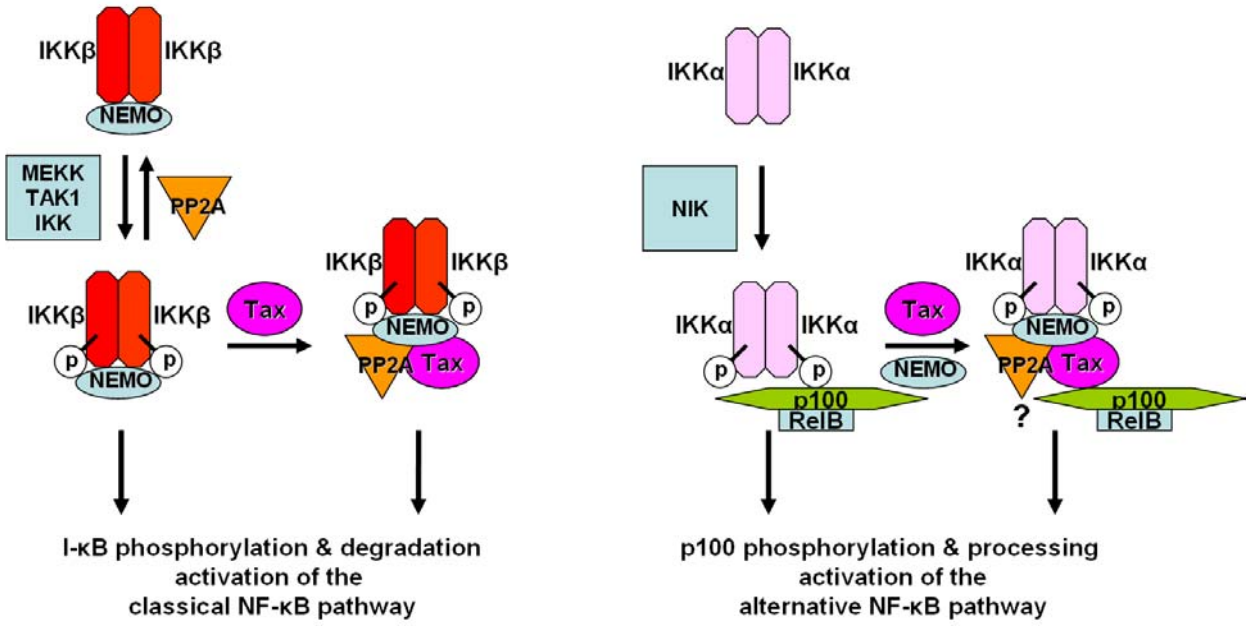

Figure 1. Schematic representation of pathways used by Tax to activate NF-κB in cells. Left, activation of the classical (canonical) NF-κB pathway; right activation of the alternative (non-canonical) NF-κB pathway.

factors—CREB and ATF-1, the 21-bp repeats, and Tax form stable ternary complexes (16-20). In these complexes, CREB/ATF-1 bind the CRE of the 21-bp repeats while Tax binds the bZIP domains of CREB/ATF-1 (21, 22, 22-24) and makes contacts with the DNA minor groove of the G/C-rich sequences that flank the CRE, thus achieving the exquisite DNA sequence specificity of LTR transactivation by Tax (25-32). In the context of the ternary complexes, Tax further recruits transcriptional co-activators, CREB binding protein (CBP)/p300 and possibly other transcription factors for potent gene activation (27, 33-37). Consistent with results from *in vitro* reconstitution, recent *in vivo* studies have indicated that a multiprotein complex that includes CREB, p300, and P/CAF is required for Tax's activation of integrated LTR (38).

5.2 Activation of NF-κB/Rel by Tax

NF-κB/Rel family of transcription factors are controlled by inhibitory I-κB proteins—I-κBα, I-κBβ, and the I-κB-like domains in NF-κB1 and NF-κB2—that sequester NF-κB/Rel in the cytoplasm as multiprotein complexes (for a recent review, see (39)). Upon activation by extracellular stimuli such as interleukin-1 (40), tumor necrosis factor-α (TNF-α), bacterial lipopolysaccharide (LPS), or by Tax, I-κBα and I-κBβ become serine phosphorylated by I-κB kinase (IKK). This marks them for polyubiquination and rapid degradation via proteasome-mediated proteolysis (Figure 1).

The holo-IκB kinase (IKK) consists of 2 catalytic subunits, IKKα and IKKβ, together with a regulatory subunit, IKKγ/NEMO (NF-κB essential modulator, referred to as IKKγ herein). In the canonical NF-κB pathway, IKKβ is both necessary and sufficient for phosphorylation of IκBα and IκBβ to effect IκB degradation and nuclear localization of NF-κB. The role of IKKα in the classical pathway is less clear. The proteasome-mediated processing of p105, the precursor to p50 NF-κB1, occurs constitutively. By contrast, the processing of p100, the precursor of p52 NF-κB2, requires activation of the non-canonical NF-κB pathway. The non-canonical pathway depends on IKKα, which phosphorylates p100 and causes its ubiqutination and proteasome-mediated processing to produce the active p52. The non-canonical pathway is important for B-cell proliferation and lymphoid organogenesis and is activated in response to a subset of NF-κB inducers such as lymphotoxin β and B cell activating factor (BAFF) (39).

Whereas different physiological inducers of NF-κB activate either the canonical or non-canonical pathway, Tax can activate both. Activation of I-κB kinase (IKK) by Tax is due in part to a direct interaction between Tax and IKKγ (41-45). Specifically, Tax binds directly to the 201-250 amino acid residues in IKKγ (46). Recent data have indicated that via a tripartite interaction, Tax, protein phosphatase 2A (PP2A) and IKKγ form a stable ternary complex. In this context, PP2A activity is inhibited or diminished (47). These results suggest that PP2A is a negative regulator of activated, phospho-IKK, and PP2A inhibition by IKKγ-bound Tax maintains IKK in a phosphorylated and active state, causing constitutive phosphorylation and degradation of I-κB, nuclear translocation of NF-κB/Rel, and potent activation of genes under NF-κB/Rel control (Figure 1).

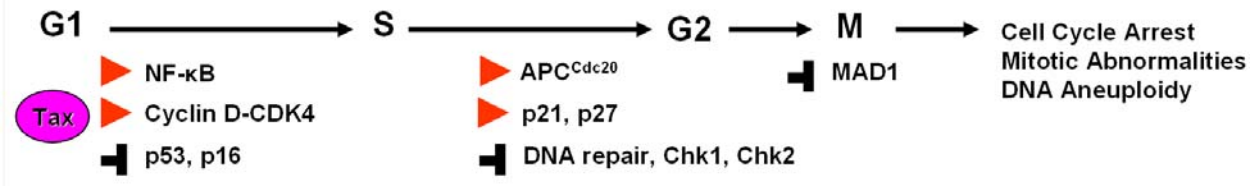

Figure 2. A summary of some of the cell cycle steps and checkpoint factors affected by Tax. This figure illustrates some examples and is not intended to be an exhaustive representation of all described Tax effects.

independent of IKKγ (48, 49). Phosphorylation of p100 by activated NIK-IKKα complex targets p100 for ubiquitination and processing (50, 51). Tax-mediated p100 processing, however, requires both IKKα and IKKγ (52-54). Tax appears to alter p100's conformation by direct binding to two short amino-terminal helices (αA and αβ) in p100 (55) and at the same time activate IKKγ/IKKα to facilitate p100 phosphorylation and processing (53, 54, 56). Because the non-canonical pathway is silent in T-lymphocytes, its aberrant activation by Tax in T-cells may play an important role in Tax-mediated T-cell activation and transformation. On the other hand, while *ex vivo* cell-transfection experiments support the importance of Tax as a potent intracellular NF-κB inducer, it should be noted that cells from ATL patients have elevated NF-κB activity even when Tax-expression is ultimately shut-down. This finding suggests that Tax may be used to initiate but may not be needed to maintain NF-κB activation (57). In fact, Higuchi et al. (58) have proposed that CD30 serves a role in Tax-independent activation of NF-κB. CD30 is a member of the TNF receptor superfamily and interestingly is a marker of malignancy in Hodgkin's lymphoma (59). Finally, NF-κB activation by Tax up-regulates many cellular genes including those of IL-2 receptor α chain, costimulatory surface receptors OX40/OX40L, IL-13, IL-15, ICAM1, and anti-apoptotic proteins such as IAP1 (60-62). The roles of these proteins in proinflammatory response and lymphocyte survival are well documented and are likely to be critical to the development of HAM/TSP and ATL.

5.3. Tax and cell cycle abnormalities

Tax perturbs critical steps of cell cycle progression [(63-73); also see (74) for review] and some of the cell cycle effects of Tax are thought to be important for cell immortalization and cell transformation (13, 14, 75, 76). Tax has been shown to activate G_1/S entry (65, 77, 78) (Figure 2). Depending on the experimental setting, Tax can induce or prevent apoptosis (79-82) or require a second stress signal in order to promote cell death (83) Tax is also implicated in inactivating the functions of tumor suppressors p53 and $p16^{INK4a}$ (84-86). More recently, Tax has been reported to (i) inhibit DNA repair (87, 88); (ii) cause clastrogenic changes and micronuclei formation (89, 90); (iii) inactivate the spindle checkpoint by binding the MAD1 protein and induce aneuploidy and formation of binucleated cells (70, 91, 92); (iv) binds a Chk2-containing complex that is involved in DNA damage checkpoint control (93); and finally, comparative studies of HTLV-1 Tax and HTLV-2 Tax have suggested that the COOH terminal region of HTLV-1 Tax may interact with PDZ-domain-containing proteins to facilitate cell transformation (94).

5.4 .Tax and spindle checkpoint

Unlike cells of other leukemia, ATL cells are often aneuploid with complex chromosomal abnormalities including trisomy 3, trisomy 7, a partial deletion of 6q, and abnormalities of 14q11 (95). Large lymphocytes with cleaved/cerebriform nuclei are also frequently seen in HTLV-I-positive individuals (96-99). These pathological findings are likely to be associated with the ability of Tax to induce formation of micro-, bi-, and multi-nucleated cells. While Tax clearly causes chromosome instability, whether this activity can be explained by a disruption of the spindle checkpoint due to Tax-HsMAD1 interaction has been questioned, in part because the initially reported HsMAD1 sequence had a nucleotide error which although leaving unchanged the first 662 N-terminal amino acid residues, frame-shifted the HsMAD1 protein sequence after residue 663, thereby extending the C-terminus to a protein of 803 residues (91, 100). Re-sequencing of the plasmids used in that study confirmed that the HsMAD1 clone used in fact correctly-encoded a 718 amino-acid-residue HsMAD1 protein, and that there was a mistake in the original sequencing. More recent findings defined the functional portion of HsMAD1 to its N-terminal sequences with C-terminal deletions retaining the checkpoint function (101). Additionally, it was reported that human spindle assembly checkpoint factors HsMAD1 and HsMAD2 were mislocated from the nucleus to the cytoplasm in HTLV-I Tax-expressing cells (102). This altered localization of HsMAD1 and HsMAD2 was thought to correlate with loss of mitotic checkpoint control. The interaction between Tax and HsMAD1 alone, however, may not fully explain the mitotic aberrations induced by Tax. Expression of *tax* in naïve cells leads quickly to multiple mitotic aberrations and, depending on experimental conditions, results in frequent losses in cell viability and proliferative capacity in many eukaryotic cells including budding yeast, *S. cerevisiae* (103). Interpretation of these results are complicated by the fact that eukaryotic yeast cells are clearly viable without a functional spindle checkpoint (e.g. a genetic deletion of MAD2); but mammalian cells without a spindle checkpoint appear to succumb to apoptotic death unless the p53 protein is also inactivated (104-108). Further studies are needed to understand better whether Tax's interaction with spindle

checkpoint and its ability to inactivate p53 function are cooperative phenomena for transformation.

We note that in some assays, *tax*-expressing HTLV-1 transformed T-cell line, MT4, continues to be sensitive to nocodazole-mediated mitotic arrest (109), suggesting that the spindle checkpoint in MT4 cells may be weakened but may not be entirely lost. That the spindle assembly checkpoint is weakened, but not completely lost, is an increasingly common finding observed for many different types of cancers (110). Finally, the cell cycle target of Tax appears to be highly conserved, since many of the Tax-induced mitotic abnormalities can be modeled in *S. cerevisiae* (109). It should be emphasized, however, that there are differences between yeast and mammalian biology, such as the absence of p53 and NF-κB functions from *S. cerevisiae*, which limits the utility of the *S. cerevisiae* system to specific aspects of cell cycle dysfunctions induced by Tax. More recently, Tax, like the HPV E6 and E7 oncoproteins, has been found to induce supernumerary centrosomes in human cells (111). This new finding suggests that more complex mechanisms beyond Tax–spindle checkpoint interaction are needed to explain aneuploidy in ATL cells.

5.5. Tax and anaphase promoting complex

Recent evidence has suggested that the mitotic pathology inflicted by Tax may be associated with unscheduled and premature activation of the anaphase promoting complex/cyclosome (APC/C, referred to as APC henceforth), a multiprotein E3-ubiquitin ligase that is required for the onset of anaphase and the exit of mitosis [see (112-116) for reviews]. The levels of cyclin A, cyclin B and the anaphase inhibitor: securin/Pds1p (precocious dissociation of sister chromatids) were found to be significantly reduced in *tax*-expressing HeLa, MT4, and *S. cerevisiae* cells (103, 109). Based on analyses of yeast mutants defective in specific APC components, the diminution of Pds1p and Clb2p brought on by Tax is thought to be mediated via APC^{Cdc20} (109).

Activation of APC during the cell cycle is mediated through its phosphorylation by Cdk1/cyclin B1 and/or polo-like kinase (Plk1), and sequential association with Cdc20 and Cdh1. Cdc20 and Cdh1 are WD40 repeat-containing proteins that are highly conserved in evolution. Both proteins function as substrate-specific activators of APC. APC^{Cdc20} is required for the onset of anaphase and APC^{Cdh1} is required for the exit of mitosis. APC^{Cdc20} becomes active during mitosis and controls metaphase to anaphase transition by targeting the destruction of critical mitotic regulators: cyclin A, securin—the anaphase inhibitor, and a subpopulation of cyclin B1 [see (112-116) for reviews].

Direct biochemical analyses now indicate that Tax directly binds human APC^{Cdc20} and activates it during the S phase, well ahead of schedule (109). This leads to the polyubiqutination and degradation of cyclin A, cyclin B1 and securin before the onset of M phase (109). Loss of these mitotic regulators during S phase is associated with delay in cell cycle progression and multiple mitotic aberrations such as DNA aneuploidy and formation of micro-, bi-, and multi-nucleated cells (72, 103, 117, 118). How the chromosome instability and other mitotic abnormalities induced by Tax impact on ATL development is not clear at present.

5.6. Tax and cell cycle arrest

Previous studies have indicated that Tax can promote quiescent T-cells to enter into G_1/S (65, 78). The potent activation of NF-κB by Tax can also be mitogenic and anti-apoptotic. In spite of these activities of Tax, constitutive expression of *tax* in most cultured mammalian cell lines is difficult to achieve. BHK, NIH3T3, and HeLa cells transduced with a *tax* retroviral vector gave rise to micro-, bi-, and multi-nucleated cells (72, 103, 117, 118). Recent evidence suggests that most of these cells are in a senescence-like state (Kuo and Giam, unpublished results). Likewise, expression of *tax* in *S. cerevisiae* leads to growth arrest and loss of cell viability (103). In agreement with these studies, Tripp et al. have reported recently that expression of *tax* in CD34+ hematopoietic progenitor cells leads to a G_0/G_1 arrest (119). Consistent with this notion, Tax has been shown to potently activate $p21^{CIP1/WAF1}$ expression previously (120-123). These results suggest that cells activated by Tax to enter G_1/S soon become arrested either immediately or after a limited number of cell divisions. Therefore, in spite of the potent mitogenic activities of *tax*, there is a strong selection against *tax* expression in previously HTLV-1-naïve cells. These phenotypes of *tax*, of course, stand in sharp contrast with those of HTLV-1-transformed human T-cell lines such as MT2, MT4, and C8166, which produce Tax abundantly, yet progress through cell cycle without any overt difficulties, albeit with delayed kinetics (103). It remains to be seen whether these cell lines harbor somatic mutations that allow them to escape the *tax*-induced senescence-like arrest and what these mutations may be. These new results also beg the question if HTLV-1 infection may lead to cell cycle arrest rather than cell proliferation as conventional wisdom has prescribed to date. Finally, it remains to be elucidated if and how some of the cell cycle aberrations caused by Tax that are outlined above may be linked mechanistically to the senescence-like G_0/G_1 arrest.

5.7. Activation of the PI3 kinase pathway and the development of ATL

When $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ levels in HTLV-1 transformed and HTLV-1 unrelated T-cells cells were compared, the former were found to express abundant $p21^{CIP1/WAF1}$, but have barely detectable levels of $p27^{KIP1}$ (124, 125). Why doesn't the over-expression of $p21^{CIP1/WAF1}$ induced by Tax lead to cell cycle arrest? What mechanism is responsible for the loss of $p27^{KIP1}$ from HTLV-1 transformed T-cells? One possible answer to the former question is that a $p21^{CIP1/WAF1}$/cyclin D2/cdk4 complex is not always an inhibitory complex and that $p21^{CIP1/WAF1}$ could potentially function as an assembly factor for the cyclin D2/cdk4 complex in HTLV-1 infected cells (126). Alternatively, it is of particular interest to note that a number of reports have implicated constitutive activation of the phosphoinositide 3-kinase (PI3K) pathway as a common feature of HTLV-1- and Tax-transformed cells. Treatment of IL-2-independent HTLV-1 transformed T-cells with inhibitors of PI3 kinase leads to a $p27^{KIP1}$-

dependent cell cycle arrest (124, 125). Transformation of Rat-1 fibroblast cells by Tax is also associated with activation of PI3K and its downstream kinase, Akt (127). Most recently, Fukuda et al. have shown that signaling through the activation-inducible lymphocyte immunomediatory molecule (AILIM)/inducible costimulator (ICOS) leads to PI3K/Akt activation, microtubule rearrangement, and formation of multi-lobulated nuclei characteristic of the flower cells seen in acute ATL (128). Finally, signaling through the PI3K/Akt pathway is known to inactivate $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ [reviewed in (129)]. Together, these reports point to the dysregulation of the PI3K pathway as a critical step in $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ inactivation, which is potentially important for escape from Tax-induced cell cycle arrest and ATL development. In the future, it will be interesting to see if activating mutations of the PI3K pathway are present in all ATL cells, and whether these mutations may constitute the early oncogenic events which allow HTLV-1-infected cells to continue to proliferate despite Tax expression, and eventually collaborate with Tax to promote leukemia development.

6. CONCLUSION

A mechanism to explain the role of HTLV-1 Tax in the etiology of adult T-cell leukemia needs to consider (a) the relative inefficiency by which HTLV-1 transforms T-cells in culture; (b) the long incubation period between virus infection and the onset of the disease; (c) the low penetrance of the disease in HTLV-1-infected population; (d) the lack of *tax* expression in ATL cells; (e) the unique karyotypic and morphological features of ATL cells; and (f) the complex and seemingly conflicting activities of Tax on G_1/S entry, DNA repair, inactivation of tumor suppressors, NF-κB activation, mitotic abnormalities, and cell cycle arrest. Recent results from many laboratories are beginning to shed lights on some of the cellular changes that may be needed to collude with Tax to cause cell transformation. It will be important in the future to see if findings from the cell-based systems can be validated in ATL cells, and to translate these basic science findings into treatments for the disease.

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