Human T Cell Leukemia Virus-I (HTLV-I) Tax-Mediated Apoptosis in Activated T Cells Requires an Enhanced Intracellular Prooxidant State

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We have shown that an estradiol-dependent activation of human T cell leukemia virus-I Tax leads to the inhibition of cell proliferation and to the induction of apoptosis. The present study demonstrates that a hormone-dependent activation of Tax promotes an enhanced prooxidant state in stably transfected Jurkat cells as measured by changes in the intracellular levels of glutathione and H2O2; these changes are followed by apoptotic cell death. Additional stimulation of the CD3/TCR pathway enhances the oxidative and apoptotic effects. Both Tax-mediated apoptosis and oxidative stress can be potently suppressed by antioxidants, as is seen with the administration of recombinant thioredoxin (adult T cell leukemia-derived factor) or pyrrolidine dithiocarbamate. Hormone-induced Tax activation induces a long-lasting activation of NF-κB, which is a major target of reactive oxygen intermediates. The long-term exposure of Jurkat cells to hormone eventually results in a selection of cell clones that have lost Tax activity. A subsequent transfection of these apparently “nonresponsive” clones allows the recovery of Tax responses in these cells. Our observations indicate that changes in the intracellular redox status may be a determining factor in Tax-mediated DNA damage, apoptosis, and selection against the long-term expression of Tax function. The Journal of Immunology, 1998, 161: 3050–3055.

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uman T cell leukemia virus (HTLV)1 is the only human retrovirus to be causatively associated with human neoplasia. A long-term persistence of HTLV-I leads to the malignant transformation of HTLV-I-infected cells that results in adult T cell leukemia (1–3). Extensive in vitro passaging of infected human T lymphocytes eventually leads to the appearance of immortalized cells with an activated phenotype. These T cells constitutively express the IL-2Rα subunit, several lymphokines such as IL-4 and IL-6, and a potent radical-scavenging and reducing agent known as adult T cell leukemia-derived factor/human thioredoxin (ADF) (reviewed in Ref. 4).

HTLV-I carries its own potential oncogene, Tax, which has no cellular counterpart (5, 6) and has been implicated in the transforming properties of the virus (reviewed in Ref. 7). Tax protein has been shown to promote the transcriptional transactivation of a wide range of growth-associated genes. In particular, it has been suggested that a Tax-induced expression of the IL-2Rα subunit promotes autocrine stimulation and consequently the transformation of HTLV-I-infected T cells (8–11). Thus far, however, only a single report has described the immortalization of human T cells through the expression of Tax alone. In this study, the immortalized T cells were particularly sensitive to activation via the CD3/TCR complex; this complex promoted their mitosis under conditions in which IL-2R function was presumably blocked (12).

A previous study using Jurkat T cells expressing a conditional version of Tax, which was generated by the fusion of the Tax protein of HTLV-I and the hormone-binding domain of the human estrogen receptor (ER-Tax), documented an inhibition of proliferation and an induction of apoptotic cell death upon the hormone-dependent activation of Tax (13). Moreover, we were able to demonstrate that caspases mediate Tax-induced apoptotic T cell death and that the CD95-signaling pathway plays a critical role in this Tax-triggered apoptotic process in our recent work using this hormone-inducible system (14). The antiproliferative effects were dependent upon the duration of ER-Tax activity and were significantly enhanced when the CD3/TCR complex was simultaneously activated (13). These observations corroborated the report of apoptotic cell death in Tax-transformed Rat-1 fibroblasts after serum starvation (15). Furthermore, the cooperative action of Tax and CD3 in T cells supports the reported overlap of the mode of action of Tax with the CD28 T cell costimulatory pathway (16).

The transcriptional complex that is activated by CD28 is closely related to NF-κB. Both play crucial roles in the TCR-independent, cyclosporin A-insensitive pathways of T cell activation. Both are known to respond to reactive oxygen intermediates (ROIs) and to be selectively suppressed by antioxidants (17, 18). Interestingly, the potent activation of NF-κB by HTLV-I Tax is, at least in transient transfection assays, also blocked by antioxidants (19). Thus, it seems likely that Tax may induce prooxidant conditions. Such redox changes are expected to function in a cooperative manner, with redox changes induced through Ag or mAb stimulation of the
CD3/TCR complex and leading to apoptosis in ER-Tax-expressing Jurkat T cells.

To test the above possibilities, we have generated ER-Tax-expressing Jurkat T cells. A hormone-dependent activation of Tax leads to the trans-activation of Tax-responsive promoters in an inducible manner. We report that a hormone-dependent posttranslational induction of ER-Tax leads to rapid changes in the intracellular levels of H$_2$O$_2$ and glutathione (GSH), promoting an intracellular prooxidant state. Concomitant activation of the CD3/TCR pathway exaggerates the oxidative stress and causes DNA fragmentation in live cells. Antioxidants block the Tax-mediated DNA damage. A long-term exposure of the cells to active ER-Tax selects for or renders surviving cells unresponsive to Tax and allows an apparent recovery of the intracellular redox status. These observations indicate that changes in the intracellular redox state may be a determining factor in Tax-mediated DNA damage, apoptosis, and unresponsiveness.

Materials and Methods

**Cell lines and transfections**

Jurkat T cells that had been stably transfected to express ER-Tax or ER-ΔTax fusion constructs were maintained in culture as described previously (13). The ER-Tax construct carried the ligand-binding domain of the human ER at the 5' end of Tax; the ER-ΔTax construct encoded an inactive fusion protein that was missing the first 12 aa of Tax. Transient transfections and chloramphenicol acetyltransferase (CAT) assays were performed by the DEAE-dextran method as described previously (13) using 3 μg of the CAT reporter plasmids pHTLV-I-LTR-CAT or p(NF-κB)-CAT and 1 μg of transactivator (ER-Tax-expressing plasmid) (13). For the induction of Tax activity, cells were treated with 1 μM of the hormone 17β-estradiol (Sigma, Deisenhofen, Germany). For the stimulation of NF-κB activity (positive control), cells were incubated with 20 ng/ml of PMA (Sigma) and 1 μg/ml of PHA (Sigma) for 1 h. Recombinant ADF was produced in *Escherichia coli* and purified as described previously (20).

**Electrophoretic mobility shift assay (EMSA)**

Total cell extracts were prepared using a high-salt detergent buffer (Totex) as described previously (21). In brief, the cells were harvested by centrifugation; washed once in ice-cold PBS; and resuspended in 4 cell volumes of Totex buffer containing 20 mM HEPES (pH 7.9), 350 mM NaCl, 20% (v/v) glycerol, 1% (w/v) of Nonidet P-40, 0.1 mM MgCl$_2$, 0.5 mM EDTA, 0.1% (w/v) EGTA, 0.5 mM DTT, 0.1% (v/v) PMSF, and 1% aprotinin. The cell lysate was incubated on ice for 20 min and then centrifuged for 5 min at 13,000 × g at 4°C. The protein content of the supernatant was determined, and equal amounts of protein (10–15 μg) were added to the binding reaction mixture containing 20 μg of BSA (Sigma), 2 μg of poly(dI-dC) (Boehringer, Mannheim, Germany), 2 μl of a buffer (20 mM HEPES (pH 7.9), 0.1% (w/v) of Nonidet P-40, 0.5 mM MgCl$_2$, 1% (w/v) PMSF, 0.1 mM EDTA, 1% (v/v) PMSF, 0.1 mM DTT, and 0.1% PMSF), 4 μl of a buffer (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, and 0.1% PMSF), and 10,000 cpm (Cerenkov) of a 32P-labeled NF-κB oligonucleotide in a final volume of 20 μl. An NF-κB oligonucleotide with a high-affinity NF-κB-binding motif (Promega, Heidelberg, Germany) was labeled with [γ-32P]ATP (3000 Ci/ mmol; Amersham, Braunschweig, Germany) and T4 polynucleotide kinase (Promega). The reaction mixture was incubated at room temperature for 25 min. Native 4% polyacrylamide gels were run in 45 mM Tris-borate, 1 mM EDTA. NF-κB-binding activity was determined by the beta-imaging of native gels.

**Apoptosis assay**

The induction of apoptosis was measured using a modified version of a method that has been described previously (22). Briefly, cells were loaded with 1 μg/ml of Hoechst 33342 (Molecular Probes, Eugene, OR) for 5 min at room temperature and then transferred on ice while protected from light before measurement. After transferring the cells to flow cytometer-compatible tubes, propidium iodide (PI) was added to a final concentration of 0.5 μg/ml. FACS Vantage (Becton Dickinson, Heidelberg, Germany) was used for sample acquisition and data analysis. Hoechst 33342 was excited at 360 nm, and emission was measured at 450 nm. PI was excited at 488 nm, and emission was detected at 610 nm. The cell population that was bright for Hoechst 33342 staining but still negative for PI was considered to be undergoing apoptosis.

**Intracellular GSH measurement**

The measurement of intracellular GSH was performed according to Rice et al. (23) with modifications. Cells that had been kept in culture medium were loaded with 20 μM of monochlorobimane (MCB) (Molecular Probes) for 10 min at 37°C. The reaction was stopped by the addition of ice-cold PBS. Cells were subsequently harvested, washed through cold FCS followed by two additional washes with cold PBS, and analyzed with a FACS Vantage flow cytometer. The 351- to 364-nm bandpass filter was used for excitation, and emission was detected at 450 nm. Dead cells were excluded by forward/side scatter gating. The mean values of the fluorescence signal from duplicate samples were considered for data analysis.

**Fluorescent measurement of intracellular peroxides with 2′,7′-dichlorofluorescin diacetate (DCFH)**

Intracellular peroxides were measured according to Royall et al. (24). A stock solution of DCFH (10 mM; Molecular Probes) was prepared under nitrogen in DMSO and stored at −20°C. Cells were loaded with 5 μM of DCFH in culture medium at 37°C for 45 min and then placed on ice before measurement. Loaded cells were collected into FACS-compatible tubes, supplemented with PI (1 μg/ml) (Sigma), and measured using a FACSScan (Becton Dickinson).

**Results**

**Stably transfected ER-Tax Jurkat cells exhibit hormone-inducible Tax activity**

ER-Tax fusion proteins were shown to transactivate several Tax-responsive reporter plasmids in a hormone-dependent manner (13). Here, we generated Jurkat T cells stably expressing inducible Tax (ER-Tax) or an inactive form of Tax (ER-ΔTax). The transactivation potential of the stably transfected, ER-Tax-expressing Jurkat cells was determined in a time-course experiment using (HTLV-I) long terminal repeat (LTR-) and NF-κB-dependent reporter plasmids. Cells were preincubated for different lengths of time in medium with or without estradiol and then transfected with the Tax-responsive reporter plasmids. The transiently transfected cells were cultivated for another 2 days in the presence or absence of estradiol before CAT reporter gene assays were performed. A hormone-dependent transactivation of the (HTLV-I)-LTR- and NF-κB-controlled promoters was observed in the cells that were cultured for 2 days with estradiol immediately following the transfections (Fig. 1, day 0). Comparable increases in CAT activity were detected with cells that had been preincubated with estradiol for 3 days (data not shown) and 7 days before transfection (Fig. 1, day −7). Thus, stably transfected ER-Tax Jurkat cells exhibit estradiol-dependent Tax-activity.

**Long-term exposure to estradiol leads to Tax unresponsiveness**

To test the long-term effects of constitutive Tax-activity, cells were preincubated for 30 days with or without estradiol before being transfected with the Tax-responsive plasmids. Preincubating the cells for 30 days with hormone led to unresponsiveness to Tax, which reduced the expression of the (HTLV-I)-LTR- and NF-κB-driven reporter gene plasmids almost to the basal level of uninduced cells (Fig. 1, day −30). Cells that had lost responsiveness to Tax due to 1 mo of continuous culture in the presence of estradiol responded to a new transient transfection of exogenous ER-Tax. In these cells, a hormone-dependent transactivation of LTR- and NF-κB-dependent reporter plasmids could be detected (Fig. 1, day −30 plus ER-Tax). Exposing control cells expressing an inactive (truncated) form of Tax (ER-ΔTax) to estradiol for the same time periods did not result in the transactivation of transiently transfected NF-κB- or LTR-CAT constructs (data not shown).

The DNA-binding activity of NF-κB was examined by EMSA for different timepoints after the activation of ER-Tax. Treating ER-Tax-expressing Jurkat cells with hormone resulted in a time-dependent, marked increase of the NF-κB-specific DNA-binding activity.
activity for \(\leq 5\) days (Fig. 2). After estradiol treatment of the cells for 5 days, a 50-fold increase of the NF-\(\kappa B\)-specific band compared with control cells was observed. Thus, in contrast to most other activators of NF-\(\kappa B\), Tax-induced NF-\(\kappa B\) activation is long-lasting and is stable for \(\geq 5\) days. In agreement with the CAT assays, NF-\(\kappa B\) activation was no longer detected after a prolonged exposure (1 mo) of the cells to hormone (Fig. 2). Control ER-\(\Delta Tax\)-expressing cells were not able to induce NF-\(\kappa B\)-binding activity at the various timepoints tested (Fig. 2 and data not shown).

**Tax induces apoptosis that is blocked by antioxidants**

We subsequently analyzed the viability of the Jurkat cells that had been exposed to Tax activity or treated with moderate concentrations (1 \(\mu g/ml\)) of anti-CD3 mAb. As shown in Figure 3, treating ER-Tax-expressing Jurkat cells with anti-CD3 mAb alone or for 30 h with estradiol alone resulted in little or no changes in apoptosis (DNA fragmentation) as measured by Hoechst 33342 staining. However, the combination of these two stimuli was strongly synergistic, leading to a nearly threefold increase in the total number of cells undergoing apoptosis compared with the background level of spontaneous apoptosis in these cells. While the induction
of apoptosis by a combination of anti-CD3 and hormone was already detectable in ER-Tax Jurkat cells at 8 h after treatment, no significant changes in the number of apoptotic cells were observed for ≥30 h posttreatment in control ER-DTax cells (data not shown). Thus, the apoptosis observed in TCR-stimulated cells could clearly be attributed to Tax activity.

To investigate the role of the intracellular redox state in Tax-mediated apoptosis, cells were pretreated with antioxidants before hormone and anti-CD3. Thioredoxin (ADF), which is a potent physiologic antioxidative protein that is able to be secreted and taken up by various cells (20, 25, 26), strongly inhibited Tax-induced apoptosis (Fig. 3). Pyrrolidine dithiocarbamate (PDTC), which is an iron chelator and radical scavenger, was slightly less effective. In ADF-treated cells, viability was even moderately increased compared with control cells. Thus, antioxidants can block Tax-induced apoptotic cell death.

**Tax induces a prooxidant state in T cells**

Since antioxidants can prevent Tax-induced apoptosis, we examined whether Tax has an effect on the cellular redox state. Jurkat cells stably expressing a hormone-inducible Tax fusion protein (ER-Tax) were treated for various times with estradiol. Untransfected cells or cells expressing ER-ΔTax were used as controls. The activation of the ER-Tax fusion protein resulted in an immediate (15 min postinduction) drop in the intracellular amount of GSH and in considerably reduced levels within 2 h after Tax activation (Fig. 4). A slow recovery of the GSH levels approaching the levels seen for control cells was seen at 5 days after the beginning of estradiol treatment. In contrast, untransfected or control ER-ΔTax Jurkat cells that had been treated with estradiol did not show any decrease in intracellular GSH levels (Fig. 4). ER-Tax Jurkat cells that were cultured in the presence of hormone for a prolonged period of time (1 mo) showed normal levels of GSH (data not shown).

To confirm the above observations, intracellular \( \text{H}_2\text{O}_2 \) levels were measured at 2 h after ER-Tax or ER-ΔTax Jurkat cells had been treated with hormone. The treatment of ER-Tax Jurkat cells with estradiol led to a marked increase in the intracellular levels of \( \text{H}_2\text{O}_2 \) and a concomitant decrease of GSH (Fig. 5, A and D). The Tax-mediated prooxidant state was inhibited by pretreating the cells with ADF or PDTC (Fig. 5, A and D). Thus, the Tax-induced...
prooxidant state as well as apoptosis can be blocked by antioxidants. These observations indicate that enhanced prooxidant conditions play a crucial role in the induction of T cell apoptosis by HTLV-I Tax.

**Tax and CD3/TCR-mediated signals synergize in inducing a prooxidant state**

The CD3/TCR complex and Tax reportedly cooperate in inducing activation-associated events in T cells as well as activation-induced apoptosis (11–13). Thus, the individual and combined effects of these two independent stimuli on the intracellular redox state of Jurkat T cells were examined. In response to treatment with immobilized anti-CD3 mAb alone, both ER-Tax- or control ER-ΔTax-expressing cells exhibited a comparable increase in the intracellular concentration of H2O2 (Fig. 5, B and C). As expected, estradiol alone enhanced the intracellular H2O2 in the ER-Tax cells, while no such increase was seen in the ER-ΔTax Jurkat cells. Treatment with anti-CD3 mAb and estradiol led to an additive enhancement of intracellular H2O2 in ER-Tax Jurkat cells, but no enhancement was observed in the control ER-ΔTax Jurkat cells (Fig. 5, B and C). While only transient changes (lasting for a few hours) were observed in the intracellular levels of H2O2 when anti-CD3 mAb was used, the changes that were induced by hormone activation of ER-Tax were sustained and lasted for several days (data not shown). This observation highlights the chronic nature of the activation events that are mediated by Tax in contrast to the transient effects that are seen in response to TCR stimulation.

**Discussion**

Our experiments indicate that Jurkat T cells stably expressing ER-Tax exhibit hormone-inducible Tax activity upon a short-term exposure to estradiol. A long-term exposure to hormone (>30 days) leads to an apparent unresponsiveness. The unresponsiveness is not due to changes in the inherent properties of the cells, as a new transfection with ER-Tax was able to reestablish hormone-dependence. One likely explanation is that cells that are defective in Tax activity have a growth advantage and are selected upon the continuous exposure of cultures to hormone. The early and late effects of Tax activation in the Tax-expressing Jurkat cells can be quite different. It has been reported previously that NF-κB activity could not be detected in the nucleus following a long-term expression of Tax in Jurkat T lymphocytes (27). In this case, the ability of mitogens and cytokines to induce NF-κB activation was also blocked. It seems that the NF-κB-signaling pathway was impaired in the latter study, since Tax was active and the induction of other transcription factors, such as Fos and Jun, was unaffected. However, our results suggest a selection against Tax activity following the prolonged activation of this protein rather than a suppression of NF-κB activation. Taken together, these observations indicate that T cells may not be able to tolerate the intracellular constitutive expression of Tax for prolonged time periods. This intolerance may explain the apparent shut down of Tax expression in HTLV-I-infected T cells.

Our posttranslational inducible system allowed a careful kinetic analysis both of the Tax-mediated effects and of NF-κB activation. We have shown previously that one of the early effects of Tax function is activation-induced T cell apoptosis (13). The observations that we report here indicate that HTLV-I Tax mediates oxidative stress, which in turn may subsequently activate NF-κB, a prooxidant-inducible transcription factor. The activation of NF-κB has been recently linked to apoptosis. NF-κB plays either an antiapoptotic or a proapoptotic role, and the role that is chosen may depend upon the cell type, subunit composition, or duration of the NF-κB response (reviewed in Ref. 28). In our study, apoptotic cell death in Jurkat cells that had been induced by Tax was shown to be accompanied by NF-κB activation. The duration of Tax stimulation determines the intensity of T cell activation and oxidative stress and the severity of the antiproliferative effect. This finding may be related in part to the persistence of a prooxidative state and to a prolonged activation of NF-κB. Transactivation and DNA-binding studies revealed that Tax activity induces a long-lasting induction of NF-κB, rather than a transient one as has been shown for several chemical and physiologic stimuli of NF-κB (27, 29). The strong NF-κB activation was entirely due to the induced Tax protein and not to a direct response of the cells to hormone, as control ER-ΔTax-expressing cells did not show any NF-κB activation after being exposed to estradiol. Therefore, as previously proposed for apoptosis triggered by serum withdrawal (30), the unusually persistent Tax-induced activation of NF-κB may exert a proapoptotic function in our experimental system.

A short-term hormone induction of ER-Tax in Jurkat cells together with the stimulation of TCR promotes apoptotic cell death (13). The apoptosis-promoting effect of Tax is particularly evident when low concentrations of anti-CD3 are used. Tax is also able to induce apoptosis in Rat-1 fibroblasts, and this apoptosis is blocked by the Bcl-2 protein (15). Interestingly, Bcl-2 has been shown to function in an antioxidant pathway to prevent apoptosis (31). Moreover, intracellular prooxidant conditions reportedly induce apoptosis in several instances (reviewed in Ref. 32). In the present study, we provide evidence that the mediator of apoptosis, Tax, can induce prooxidant conditions in the cell. This is revealed by a decrease in the intracellular GSH level that occurs rapidly (within 15 min) and lasts for several days. The decrease in the intracellular GSH level is accompanied by a Tax-mediated increase of intracellular H2O2 levels. The long-lasting, Tax-induced prooxidant state correlated with the observed apoptosis and NF-κB activation. Tax-induced NF-κB activation can be blocked by antioxidants (19). In addition, Tax-induced apoptosis can be prevented by radical scavengers.

The fact that Tax activity induces a prooxidant state in the cell shows that Tax uses common pathways to induce cellular activation and apoptosis. Since ROIs are involved in the induction of CD95 ligand (CD95L) mRNA expression in some cases (33, 34), this involvement could explain the mechanism underlying the Tax-induced up-regulation of CD95L (14). Moreover, it has been recently shown that prooxidant-induced NF-κB activation may be involved in the transcriptional activation of the CD95L gene (34, 35).

Tax can transactivate the antioxidative stress protein ADF/thioredoxin (36). ADF is a stress-inducible protein that is secreted from cells; this protein plays an essential role in cellular protection against oxidative stress and cell death and is itself induced by various oxidative agents (37).

The present work provides direct evidence for the production of ROIs by Tax and the involvement of these intermediates in Tax-induced apoptotic T cell death. Our findings suggest that changes in the intracellular redox status may be a critical factor in Tax-mediated DNA damage and apoptosis and in the apparent shut down of Tax expression in HTLV-I-infected cells.

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References


