Role of the Fas/Fas ligand pathway in apoptotic cell death induced by the human T cell lymphotropic virus type I Tax transactivator

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Two distinct human diseases have been described in association with human T cell lymphotropic virus type I (HTLV-I) infection: adult T cell leukaemia and tropical spastic paraparesis/HTLV-I-associated myelopathy. Although comprehensive understanding of specific mechanisms underlying pathogenesis of either disease has not yet been achieved, the viral regulatory protein Tax is believed to play a significant role. Previous studies demonstrated the potential of Tax to transform host cells. Here, it is shown that the Tax transactivator has in addition the potential to induce T cell death by apoptosis. Using an inducible system (Jurkat cell line JPX-9), significant apoptotic cell death upon Tax expression was observed. In an attempt to detect the cellular genes mediating this effect, it was found that induction of Tax was associated with marked up-regulation of the Fas ligand (FasL) gene. Tax-induced apoptosis was inhibited when the Fas/FasL pathway was interrupted by YVAD-cmk, the inhibitor of ICE-like proteases. Transient expression experiments provided additional support for the putative role of endogenous FasL in Tax-induced apoptosis. Upon cotransfection with Tax-expressing plasmid, the transcriptional activity of the FasL promoter was found to be significantly upregulated in Jurkat cells and several other cell lines, as measured by reporter gene expression. Furthermore, cotransfection using different Tax mutants demonstrated that both CREB and NF-κB activation domains of Tax protein were required for the transactivation to take effect.

Introduction

Mechanisms involved in the pathogenesis of human T cell lymphotropic virus type I (HTLV-I) infection have been subject to intensive research in recent years. Although several viral proteins have been proposed to be involved, the prevailing body of data suggests a major role for the transactivating protein Tax (Green et al., 1989; Grossman et al., 1995; Nerenberg et al., 1987, 1991). The Tax transactivator is a 40 kDa product of the pX genomic region located in the proximity of the 3’ end long terminal repeat (LTR). Tax is indispensable for the virus life cycle by virtue of its effect in trans on LTR transcriptional activation (Cann et al., 1985; Sodroski et al., 1984). The effect of Tax is not mediated through direct interaction with the transcriptional regulatory elements in the promoter region; rather, it is accomplished through at least three host-related transcription factor pathways. They include members of the cAMP response element binding protein and activating transcription factor family (CREB/ATF) and NF-κB/Rel proteins (Arima et al., 1991; Fujii et al., 1992; Leung & Nabel, 1988; Maruyama et al., 1987; Zhao & Giam, 1992). As a consequence, the Tax protein appears to be a powerful transcriptional transactivator of a variety of other viral and cellular genes, most importantly human immunodeficiency virus (HIV) LTR, IL-2, IL-2Rα, GM-CSF, and c-fos (Inoue et al., 1986; Nagata et al., 1989; Nimer et al., 1989; Zimmermann et al., 1991). Such pleiotropic activity might result in the widespread dysregulation of gene expression believed to form the basis of HTLV-I pathogenic potential (Smith & Greene, 1991).

Although the previously described transactivating effects of Tax may bear specific relevance to direct neoplastic transformation of T cells, the induction of genes involved in
Fig. 1. Induction of cell death in Jurkat cells by Tax transactivator. (a) Jurkat JPX-9 cells, stably transfected with the inducible \textit{tax} gene, and control JPX/M cells, harbouring a nonfunctional mutant of the \textit{tax} gene, were induced to express the Tax protein with 10 µM CdCl\(_2\). Cell viability was determined by the trypan blue dye exclusion method just prior to induction and later at 24 h intervals. (b) The distribution of the cells undergoing different stages of cell cycle (G\(_0\)/G\(_1\), S and G\(_2\)) was determined by flow cytometric analysis. At the time of Tax induction, and 24 and 48 h later, cells were stained with propidium iodide and analysed in a Becton Dickinson FACStar Plus.
apoptosis is another aspect that warrants investigation. Notably, in the face of a high rate of apoptosis among adult T cell leukaemia (ATL) cells in vitro, the notion that one or several HTLV-I gene products may be involved in this process seems plausible (Tsuda et al., 1993). The present study was undertaken to delineate functional involvement of Tax in apoptosis using a Tax-inducible CD4+ T cell line (Jurkat JPX-9) as a model.

**Methods**

**Cells lines and induction of Tax expression.** Jurkat cells (TIB 152; American Type Culture Collection, Rockville, Md, USA), and Tax-inducible JPX-9 and control JPX/M cells (Nagata et al., 1989) were grown to a density of 5 × 10^6 cells/ml in 1640 RPMI medium supplemented with 10% foetal calf serum, glutamine and antibiotics. Expression of biologically active Tax or a non-functional Tax mutant was induced by addition of CdCl2 to 10 µM final concentration. Following induction, the medium containing CdCl2 was replaced every 24–36 h and the cultures were maintained for a period of 6 days. Aliquots were withdrawn at regular 24 h intervals and, after viability had been determined by the dye exclusion method, the cells were further processed for DNA fragmentation analysis or RNA isolation.

**Analysis of DNA fragmentation by agarose gel electrophoresis, flow cytometry and electron microscopy.** For analysis by electrophoresis in agarose gel, 5 × 10^6 cells were processed to obtain the cytosolic DNA. Cells were first lysed with 10 mM Tris/HC1 pH 7.5, 0.2% Triton X-100, and after removal of nuclei by centrifugation, the cytosolic fraction was extracted with phenol–chloroform–isoamyl alcohol (25:24:1) and treated with RNase A (0.6 mg/ml) at 37 °C for 30 min. After precipitation, the samples were electrophoresed in a 2% agarose gel and visualized by ethidium-bromide staining.

Cells used in the flow cytometric analysis were washed with PBS and fixed with 70% ice-cold ethanol for 10 min. After fixation, the cells were washed once again, resuspended in PBS with 0.1% propidium iodide and finally treated with RNase A (100 units/ml) for 30 min at room temperature (Nicoletti et al., 1991). Individual samples (10^6 cells) were then analysed in a FACStar Plus (Becton Dickinson) using a 488 nm argon laser for excitation and gating on forward versus side scatter.

For electron microscopy, cells harvested 48 h after CdCl2 induction were fixed with glutaraldehyde–OsO4, stained with uranyl acetate–lead citrate, and examined with a Phillips CM10 transmission electron microscope at 80 kV.

**Inhibition of Tax-induced apoptosis.** 5 × 10^3 JPX-9 cells were seeded per well in a 24-well plate and incubated with 600 nM IL-1β converting enzyme (ICE)-like inhibitor Acetyl-Tyr-Val-Ala-Asp-chloromethylketone (YVAD-cmk, Calbiochem) for 2 h, then 10 µM CdCl2 was added to induce tax expression. After 48 h incubation, the cells were harvested for DNA preparation as described previously (Liu et al., 1995). In parallel, the cells were treated with 250 ng/ml anti-Fas antibody (clone CH-11, MBL) and a 1 µg DNA aliquot was labelled with [α-³²P]dCTP using Rosl's method for detection of apoptosis (Rosl, 1992). The electrophoretic patterns of chromosomal DNAs were visualized with InstantImager (Packard).

**Northern blotting and RT–PCR.** Total RNA was isolated using the acid–phenol extraction method (Chomczynski & Sacchi, 1987; samples of 5 µg were then fractionated in formaldehyde agarose gels and transferred to PVDF-N membranes (Millipore). The random-primed hybridization probes specific for tax, Fas and Fas ligand (FasL) genes were prepared using pSGTax (provided by Dr M. Seiki, Kanazawa University, Japan), pBLF58-1 (Itoh et al., 1991) and pBX-hFHL1 (Takahashi et al., 1994) plasmids, respectively. Hybridization was performed according to the standard protocol (Sambrook et al., 1989). To control for the specificity of Tax mRNA detection, the expression of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene was determined using a commercially available probe (Clontech).

In order to detect the expression of Fas and FasL genes at the transcriptional level by PCR, 0.2 µg of poly(A)+ RNA was reverse-transcribed using SuperScript II RNase H – Reverse Transcriptase (Life Technologies) in 20 µl final volume. PCR amplification was then performed using 1/10 of the first strand cDNA yield. The optimal amplifiers were designed using PrimerSelect of the program package Lasergene (DNASTar); for the Fas-specific amplification the upstream 5’ GAGTGAGGAAGGGCTTTACGAGT and downstream 5’ AGGGACCCAGTCTGGTTCAATC primers were selected to bind a 650 bp long amplicon, and for the FasL amplification the amplicon (343 bp) was determined by the upstream 5’ GAATTGGCCTGGGATTGTTCA and downstream 5’ TGGCAGTCTGGGAGTTG primers. After 30 cycles, the PCR products were separated in 1% agarose gel, transferred to PVDF-N membrane and hybridized with Fas- and FasL-specific probes (provided by Dr Nagata, Osaka Bioscience Institute, Osaka, Japan) by Northern blot analysis as described above.

**Construction of the Fas ligand reporter plasmid and transient expression assay.** The human FasL promoter has been previously cloned in the pBL-hFL5H3-1 plasmid (Takahashi et al., 1994). In order to enable subcloning and subsequent analysis of the promoter, we determined the promoter sequence by automated cycle sequencing with dye primers (Thermo Sequenase sequencing kit, Amersham) using the ALFexpress system (Phamacia). The 1508 bp FasL promoter was then amplified using the 27 bp forward primer 5’ GAAGATCTCCATGTTTACCTTGTTTTCACTTGGCAGTCTGGGAGTTG and subcloned into the pGL3-Basic luciferase (Luc) reporter plasmid (Promega) (pFasL-luc). FasL transcriptional activation was then tested by cotransfecting Jurkat cells with the Tax-expressing plasmid pCMVTax and a series of Tax mutant plasmids pCMVM7 (defective in induction of both NF-κB and CREB pathways), pCMVM22 (defective in induction of the NF-κB pathway) and pCMVM47 (defective in induction of the CREB/ATF pathway) (provided by Dr W. Greene, Gladstone Institute of Virology and Immunology, San Francisco, USA) (Smith & Greene, 1990). All transfection experiments were performed in triplicate by applying a preformed mixture containing 4 µl liposome reagent DMRIE-C (Gibco-BRL) and 2 µg of each plasmid to 2 × 10^5 cells for 5 h. The transfection was terminated by adding fresh medium and the cells were further cultured for 48 h, after which luciferase was extracted according to the manufacturer’s instructions (Promega). Luc activity was determined by a bioluminescent assay using an automated microplate luminometer (Luminoscan, Labsystems). Light output in individual samples was normalized on the basis of protein content, which was determined by the Bio-Rad DC protein assay (Pierce). The transfection of JEG-3 cells was described previously (Zachar et al., 1994). Tax-inducible JPX-9 cells were transfected with a single plasmid only, the Fasl reporter construct or control pGL3-Basic plasmid. The transfection procedure was identical to that described above for Jurkat cells, except that after transfection cells were grown in the presence of 10 µM CdCl2.

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Results

Induction of apoptosis in JPX-9 cells by Tax and Fas-specific antibody

To investigate the effect of Tax on apoptosis, we used the CD4+ Jurkat cell lines JPX-9 and JPX/M stably transfected with the wild-type and mutated tax genes, respectively, under the control of the inducible metallothionein promoter (Nagata et al., 1989). In JPX/M cells the tax gene was rendered inactive by introducing a frameshift mutation in the vicinity of the 3' end of the reading frame. The induction of Tax was achieved with CdCl₂, which was added to cultures at the minimum concentration (10 µM) sufficient to obtain effective induction of Tax and still show no cytotoxic effect.

The expression of Tax protein proved to have a dramatic effect on cell viability. While JPX/M cells grew exponentially over a period of 5 days after induction, the number of JPX-9 cells decreased gradually, with only 15% cells remaining at the end of experiment (Fig. 1 a). In line with these data, the flow cytometric analysis demonstrated profound changes in the distribution of cells in all stages of cell cycle (Fig. 1 b). The proportion of living JPX-9 cells, expressed as a sum of the cells in G₀/G₁, S and G₂ phases, diminished from 75% to 32±6% in the 48 h after induction, whereas in the control JPX/M cells this proportion changed negligibly, from 87±9% to 82±2%.

In the ensuing experiments, we aimed to characterize structural changes in the chromosomal DNA which were associated with Tax-induced cell death. After induction with CdCl₂, gel electrophoretic analysis demonstrated a ladder banding pattern suggestive of internucleosomal DNA fragmentation characteristic of apoptosis exclusively in the JPX-9 cells (Fig. 2 a). Furthermore, functionality of the Fas-specific apoptotic pathway was verified by parallel treatment of JPX/M and JPX-9 cells with anti-Fas antibody (250 ng/ml, clone CH-11). After 9 h, significant fragmentation of chromosomal DNA was detectable in both cell lines by gel electrophoresis (Fig. 2 b).

Transmission electron microscopy provided further evidence for apoptosis in JPX-9 cells (Fig. 2 c). A characteristic morphological picture with prominent nuclear condensation and segmentation, and cytoplasmic vacuolization became apparent 48 h after Tax induction. None of these changes could be seen in control JPX/M cells.

Tax-induced expression of the FasL gene in JPX-9 cells

The pleiotropic effect of Tax on gene modulation has been proposed to be mediated through regulation of major transcriptional pathways. Apoptosis could be triggered in response to Tax by one or several genes directly involved in programmed cell death. Previous reports (Nagata et al., 1989) and our own studies (unpublished data) suggest that transcriptional expression of TNF-α, c-Myc, Bcl-2, IRF-1, IRF-2 or ICE is not affected by Tax in JPX-9 cells. However, the Fas/FasL pathway which was found to induce apoptosis as a result of phorbol myristyl acetate (PMA) and ionomycin stimulation or T cell receptor engagement in T lymphocytes (Alderson et al., 1995; Dhein et al., 1995; Suda et al., 1995) could also be operative in Jurkat cells. The representative results from RT–PCR analysis of FasL expression in JPX-9 and JPX/M cells demonstrated that specific transcripts first became detectable at 4 h and reached maximum levels 24 h after Tax induction in JPX-9 cells (Fig. 3). Notably, the Fas gene remained expressed at constitutive levels and did not respond to Tax transactivation. Cumulatively, these data provided initial...
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Fig. 3. Expression of Fas and FasL at the transcriptional level in JPX-9 cells after induction of Tax transactivator. JPX-9 cells expressing Tax and control JPX/M cells expressing a nonfunctional mutant of Tax were analysed for Fas and FasL expression by RT–PCR at the indicated intervals after induction with 10 µM CdCl₂. The amounts of Tax- and G3PDH-specific transcripts were determined by Northern blotting. The expression of the G3PDH gene was determined for standardization purposes.

Inhibition of Tax-induced apoptosis by the ICE inhibitor YVAD-cmk

To establish the causal role for Fas/FasL in Tax-induced apoptosis we investigated the effect of inhibition of ICE-like proteases which have been found indispensable for Fas/FasL to exert its effect (Enarl et al., 1995; Los et al., 1995). The process through which activity of ICE-like proteases are stimulated after Fas activation is apparently not dependent on de novo synthesis of RNA and can be specifically inhibited by YVAD-cmk. Previously, this inhibitor has been shown to block efficiently apoptosis which was triggered by anti-Fas antibody in cultures of Jurkat cells (Enarl et al., 1995).

By using YVAD-cmk, we were able to inhibit completely Tax-induced apoptosis, as is indicated by decreased DNA fragmentation in Fig. 4(b). In the parallel experiment in which apoptosis was induced by anti-Fas antibody CH-11 (250 ng/ml), similar inhibition was obtained (Fig. 4a). These data thus provide strong evidence for involvement of the Fas/FasL pathway in Tax-induced apoptosis.

Transactivation of the FasL promoter by Tax

In an attempt to determine the extent to which Fas ligand expression is subject to Tax regulation, we performed a series of transient transfection assays. To this end, we employed...
Fig. 5. Nucleotide sequence of the human FasL promoter region. The numbering system starts at the last base of the ATG initiation codon and the potential cis-acting elements are underlined.

pFasL-luc, a reporter construct featuring the Luc gene under the control of the 1508 bp FasL promoter sequence (Fig. 5). In addition to the motifs present in the 500 bp region previously characterized by Takahashi et al. (1994), the upstream 1 kb segment revealed numerous cis-regulatory transcriptional elements. Upon cotransfecting with pCMVMTax, expressing the wild-type tax gene, in Jurkat and JEG-3 cells the basal activity of FasL promoter increased 26- and 17-fold, respectively (Fig. 6). Moreover, similar results were obtained when several other non-lymphocytic cell lines (COS-7, NIH-3T3 and SK-N-SH) were used to test the effect of host cell-specific factors on FasL transactivation by Tax (data not shown). In addition, activation of the FasL promoter comparable to that in Jurkat cells could also be seen in the JPX-9 cells which were induced to express Tax using CdCl₂ (data not shown).

A further series of transient expression experiments was aimed to determine in more detail the molecular basis of Tax interaction with the FasL promoter. The Tax protein exhibits two discrete domains, each involved in activation of a specific transcriptional regulatory pathway. One of these pathways is mediated via the CREB/ATF family of transcription factors, the other via the Rel-related family of κB enhancer-binding proteins (Arima et al., 1991; Fujii et al., 1992; Leung et al., 1988; Maruyama et al., 1987; Zhao & Giam, 1992). We employed Tax variants which were defective in NF-κB (pCMVM22), CREB/ATF (pCMVM47) or both NF-κB and CREB/ATF (pCMVM7) activation domains (Smith & Greene, 1990) to explore the transcriptional pathways.

A mutation within the putative zinc finger domain which rendered Tax inactive in both CREB/ATF and NF-κB pathways (M7) totally abolished the transactivating effect of Tax in Jurkat cells (Fig. 6). Moreover, the mutant M22 with the CREB/ATF but not NF-κB activity exhibited about 27% of the full Tax transactivation potential, and the Tax mutant M47 which retained NF-κB but not CREB/ATF activity showed only 12% of Tax transactivation activity. Importantly, however, the original level of Tax transactivation could be restored in a synergistic fashion with the M22 and M47 mutants being co-expressed. Similar results were obtained when these experiments were repeated using JEG-3 as host cells. Here again, both M22 and M47 variants proved completely inactive in transactivation, whereas the original
activity could be restored upon their co-expression (Fig. 6b). Thus it appears that an effect mediated by both activation domains is indispensable for effective FasL transactivation by Tax.

**Discussion**

Infection with HTLV-I and the associated pathogenesis has been subject to growing interest in recent years, but a comprehensive understanding of the pathogenic mechanisms is still elusive. In an effort to define a role for viral proteins in development of the disease process, attention has been focused on the Tax transactivator. Tax has a potential to transactivate numerous early response genes that are normally transiently induced following antigenic or mitogenic activation of T cells (Kelly et al., 1992). Thus it appears plausible that prolonged transactivation by Tax might result in cell immortalization. In addition, the demonstration of neoplasms of mesenchymal origin in tax-transgenic mice appears to further support the pathogenic role of Tax (Green et al., 1989; Grossman et al., 1995; Nerenberg et al., 1987, 1991). However, transformed T cells have never been observed in these experiments despite the fact that CD4+ T cells are a major target for HTLV-I during infection in vivo. Observations from patients with ATL also seem to challenge the importance of Tax for malignant transformation. Intriguingly, Tax was found to be expressed in these patients at significantly lower levels than in asymptomatic carriers or tropical spastic paraparesis/HTLV-I-associated myelopathy patients (Furukawa et al., 1995; Gessain et al., 1985). Furthermore, the latest data show that HTLV-I gene expression is not indispensable for the maintenance of neoplastic cell growth in the culture and that expression of the tax gene is not sufficient to induce neoplasia in hu-SCID mice (Imada et al., 1995).

Recent evidence suggests that, in addition to its transforming capacity, the Tax protein may also be involved in triggering apoptotic cell death. Initially, apoptosis was observed in cultures of primary ATL cells and Tax-transformed Rat-1 fibroblasts as a result of serum deprivation (Tsuda et al., 1993; Yamada et al., 1994). Subsequently, Chlichlia et al. (1995) demonstrated apoptosis in T cells expressing Tax following CD3 stimulation. In the present report, we have shown that Tax alone can induce apoptotic cell death in the CD4+ Jurkat T cell line and, furthermore, we have provided evidence that implicates the Fas/FasL pathway in this process.

The Fas/FasL pathway is thought to play a central physiological role in the control of immunological homeostasis through elimination of mature or activated T cells and maintenance of self-tolerance (Alderson et al., 1995; Nagata & Golstein, 1995; Zheng et al., 1995). In the light of our findings, it is plausible that the Fas/FasL pathway also plays a prominent part in the pathogenesis of ATL. Fas antigen is preferentially expressed on the surface of CD4+ and CD45RO+ cells which are a major target for in vivo HTLV-I infection, thereby providing a receptor pool which effectively may be activated by FasL induced as a result of virus infection (Debatin et al., 1990; Miyawaki et al., 1992; Nagatani et al., 1990; Richardson et al., 1990; Shirono et al., 1989; Worner et al., 1990). Elimination of infected cells through apoptosis triggered by Fas–FasL interaction could provide an explanation for long-term latency of HTLV-I infection and a characteristically low frequency of cells expressing virus in infected individuals. On the other hand, the malignantly proliferative T cell phenotype which expresses high levels of Fas antigen (Debatin et al., 1990)
would be expected to be defective in FasL expression. Indeed, our analysis of multiple HTLV-I-transformed T cell lines failed to provide any evidence of FasL expression (unpublished observations).

Strikingly, the control of FasL induction by Tax appears to be accomplished through a series of regulatory interactions. Although the data presented clearly implicate the NF-κB and CREB/ATF activation pathways of the Tax transactivator, they do not provide a complete picture of the mechanisms involved. The absence of binding sites for NF-κB and CREB/ATF transcription factors suggests that intermediate genes are induced in order to interact with the FasL promoter.

In conclusion, the significance of mechanisms eliciting apoptotic cell death during HTLV-I infection should be comprehensively explored. By the same token, more advanced studies of the elements responsive to Tax transactivation in the FasL promoter are warranted.

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