Human T cell leukemia virus type-I Tax activates human macrophage inflammatory protein-3α/CCL20 gene transcription via the NF-κB pathway

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Abstract

Infection by human T cell leukemia virus type (HTLV)-I is associated with several diseases, including adult T cell leukemia and HTLV-I-associated myelopathy/tropical spastic paraparesis. Leukocytes are attracted to the sites of inflammation by chemotactic factors. Macrophage inflammatory protein (MIP)-3α/CCL20 is a recently isolated member of the CC subfamily of chemokines and has been proposed as a crucial factor to elicit inflammatory reactions. We now report that endogenous MIP-3α mRNA levels are elevated in HTLV-I-infected T cell lines and in a human T cell line following the induced expression of the HTLV-I-encoded transactivator, Tax. Analysis of the human MIP-3α promoter revealed that this gene is activated by Tax, via the activation of nuclear factor (NF)-κB, whose responsive element, –82-κB, is located at a position between –82 and –91 relative to the putative transcription start site. With an electromobility shift assay we further demonstrated that the –82-κB element was bound by the Tax-activated p50/p65 heterodimers of NF-κB. Expression of the specific receptor of MIP-3α, CCR6, was also increased in HTLV-I-infected T cell lines, suggesting an autocrine and/or paracrine mechanism to establish the pathogenesis of HTLV-I-associated diseases.

Introduction

Human T cell leukemia virus type (HTLV)-I is the etiological agent of adult T cell leukemia (ATL) (1,2), a mature T cell-derived neoplasia with a highly invasive phenotype. The involvement of HTLV-I has been also suggested in several chronic inflammatory diseases, such as HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (3,4), HTLV-I-associated arthropathy (5), uveitis (6) and Sjögren’s syndrome (7).

The HTLV-I genome encodes several accessory molecules, including a 40-kDa protein, Tax. Tax is important to transform cell lines and primary T cells in vitro (8–11). Transgenic mice expressing Tax developed tumors, including those of a non-lymphoid origin and various inflammatory diseases (12–15). These multiple functions of Tax are mainly attributable to its trans-acting activity in the transcription of a number of cellular genes as well as the HTLV-I long terminal repeat. The transactivation by Tax is mediated through interactions with transcription factors, such as cAMP-responsive element binding protein (16), NF-κB (17) and serum-responsive factor (CarG box binding protein) (18).

A characteristic feature of the HTLV-I-associated diseases is the infiltration of lymphocytes, including infected cells, into the affected lesion. In general, the infiltration of cells is dependent on chemotactic factors, such as C5a of the complement system, N-formylated oligopeptides of bacterial origin, intermediates of lipid metabolism and chemokines. Macrophage inflammatory protein (MIP)-3α/CCL20, also known as LARC and Exodus, is a recently identified chemokine...
that chemotacts subsets of lymphocytes and dendritic cells through its specific receptor, CCR6 (19–26). It has been proposed that MIP-3α has an important role in the recruitment of dendritic cells and lymphocytes to the site of inflammation (27,28), and in the regulation of inflammation (29).

In the present study, we report that HTLV-I-infected T cell lines constitutively express MIP-3α mRNA and that Tax is capable of inducing MIP-3α gene expression in uninfected T cells. We demonstrate that Tax-induced MIP-3α transactivation is mediated through an NF-κB-binding site, located 82 bp upstream from the putative transcription start site of the MIP-3α gene. We further demonstrate that the mRNA of CCR6, a receptor for MIP-3α, is also strongly expressed in HTLV-I-infected T cells.

**Methods**

**Cell culture and RT-PCR**

The Jurkat, MOLT4, HUT102, MT-2, KK-1, ST-1 (30–32) and JRX-9 (kindly provided by Dr. M. Nakamura, Tokyo Medical and Dental University, Tokyo, Japan) cell lines were cultured as previously described (33). Total RNA was extracted by using the RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. The first-strand cDNA was synthesized using an RT-PCR kit (Stratagene, La Jolla, CA) with oligo(dT) primers. Thereafter, the cDNA was amplified for 30 cycles for MIP-3α, CCR6 and Tax, and 23 cycles for β-actin. The oligonucleotide primers used in the experiments were as follows: for MIP-3α, sense 5′-ATGTCGTTACCAAGGTTTGC-3′, antisense 5′-CCAATTCCTACAGAAAA-CC-3′; for CCR6, sense 5′-CTGAGATGTTACTGTGCC-3′, antisense 5′-TTTTCGTCGCGGTATGTTT-3′; for Tax, sense 5′-GGTACCCAGTCTACGTTT-3′, antisense 5′-AGAGTATCATGACACACG-3′; for β-actin, sense 5′-AAGAGGACCTGTCCTACG-3′, antisense 5′-TACATCGCTGGGGTGTTG-3′. Cycling conditions were as follows: denaturing at 94°C for 60 s, annealing at 55°C for 60 seconds for MIP-3α or CCR6, and at 60°C for 60 s for Tax or β-actin respectively, and extension at 72°C for 60 s.

**ELISA**

MIP-3α was measured in supernatants using the Quantikine human MIP-3α immunoassay kit (R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The lower limit of detection was 0.47 pg/ml.

**Plasmids**

The pGL2-MIP-3α luciferase vector was constructed by cloning the promoter region, from −871 to +58 of the human MIP-3α gene, into a promoterless luciferase reporter plasmid, pGL2-basic (Promega, Madison, WI) (Fig. 5A). The pGL2-MIP-3α/xkB vector has an inactivated NF-κB-binding site located 82 bp upstream of the putative transcription start site of the MIP-3α promoter (Fig. 5A). The pNFKB-Luc vector, containing five tandem repeats of an NF-κB-binding site, was purchased from Stratagene. The pRL-β-lact vector expresses the renilla luciferase under the control of the human β-actin promoter (33). The pcDNA3-IκBαSR vector was constructed by cloning the super repressor form of IκBα, bearing a serine-to-glycine substitution at residue 32 and a serine-to-alanine substitution at residue 36 of human IκBα, into the expression vector pcDNA3 (Invitrogen, Carlsbad, CA). The pLTEN plasmid, a Tax expression vector, and its empty vector, pLXEN, were kindly provided by Dr T. Akagi (Osaka Bioscience Institute, Osaka, Japan) (34).

**Cell transfection and luciferase assay**

Transfections were performed by using the FuGENE6 transfection reagent (Roche, Indianapolis, IN) for HeLa cells, and by using the TransFast transfection reagent (Promega) for Jurkat, HUT102 and JRX-9 cells, according to the manufacturer’s protocols. In all cases, pRL-β-lact was co-transfected to correct for transfection efficiency. The luciferase activity was measured as previously described (33).

**Electrophoretic mobility shift assay (EMSA)**

EMSA was performed as previously described (33). Briefly, an aliquot of the nuclear extracts (5 µg) from JRX-9 cells, incubated in the absence or presence of 130 µM of ZnCl₂ for 24 h, was incubated in a reaction buffer (10 mM HEPES, pH 7.9, 100 mM NaCl, 0.4 mM MgCl₂, 0.3 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol and 100 µg/ml poly[dI–dC]). The 32P-end-labeled, double-stranded oligonucleotide containing the sequence of the MIP-3α promoter with the putative NF-κB site (5′-CACATGGGTGTTTCCCCATTGA-3′) was used as the probe. The same, unlabeled oligonucleotide or the mutant sequence (5′-CACATGTTTCCCCATTGA-3′) was used as a competitor. For a typical NF-kB competitor, the sequence (5′-GGGCTGGGATCCTCCGTAA-3′) of the IFN regulatory factor (IRF)-1 promoter was used (33).

**Flow cytometric analysis**

For the analysis of the CCR6 cell surface expression in the HTLV-I-positive and -negative T cell lines, cells were washed with staining buffer (PBS, 0.2% BSA), and then incubated for 30 min on ice with FITC-conjugated anti-human CCR6 antibody (R & D Systems) or isotype-matched control antibody (PharMingen, San Diego, CA). After three washes with staining buffer, the cells were suspended in staining buffer and were analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) with the single-color staining method after gating on forward and side scatter to exclude debris and clumps, using CellQuest software (Becton Dickinson).

**Results**

MIP-3α mRNA is constitutively expressed in T cell lines infected with HTLV-I

To determine whether MIP-3α expression in T cells correlates with HTLV-I infection, the expression of MIP-3α mRNA was analyzed by RT-PCR using MIP-3α-specific primers. The HTLV-I-positive T cell lines, HUT102 and MT-2, strongly expressed MIP-3α mRNA (Fig. 1A, top, lanes 3 and 4), while ATL-derived cell lines, KK-1 and ST-1, weakly expressed MIP-3α mRNA (data not shown). On the other hand, the HTLV-I-negative T cell lines, Jurkat and MOLT4, did not express this mRNA (Fig. 1A, top, lanes 1 and 2). The expression of Tax mRNA was confirmed in HUT102 and MT-2 (Fig. 1A, middle).
KK-1 and ST-1, Tax mRNA is detected, but at very low levels, as previously reported (32,35). Furthermore, the analysis of MIP-3α protein expression by ELISA confirmed that this chemokine is produced by the HTLV-I-positive T cell lines (Fig. 1B). These results demonstrate that MIP-3α is constitutively expressed in HTLV-I-infected T cell lines.

Tax induces MIP-3α gene expression

We next examined whether Tax up-regulates the MIP-3α expression in T cells, because Tax has been reported to be involved in the deregulation of host genes and the data presented above showed that the expression profile of MIP-3α seemed to correlate with that of Tax. We used the inducible Tax transfectant of Jurkat, JPX-9, which produces Tax after the addition of CdCl₂ or ZnCl₂ (36,37). As analyzed by RT-PCR, the expression levels of MIP-3α is detected in 3 h and maintained up to 24 h, and this kinetics correlated well with the induced expression of Tax (Fig. 2). These results suggest that Tax is capable of inducing the expression of the MIP-3α gene in Jurkat T cells.

MIP-3α promoter activity is induced by Tax

To examine whether the MIP-3α promoter responds to Tax, we co-transfected a Tax-expression plasmid together with the luciferase reporter directed by MIP-3α 5′-regulatory sequences (pGL2-MIP-3α). As shown in Fig. 3, Tax caused a remarkable induction of the pGL2-MIP3α reporter construct in HeLa cells (Fig. 3A). In addition, when JPX-9 cells were transfected with pGL2-MIP-3α, the reporter activity was specifically induced in the cells incubated with ZnCl₂, but not in those incubated in the absence of ZnCl₂ (Fig. 3B). These results indicate that Tax transactivates the MIP-3α promoter activity in cells of different origins.

Tax transactivates the MIP-3α promoter via the NF-κB pathway

It has been reported that the NF-κB pathway is important in the tumor necrosis factor (TNF-α)-mediated MIP-3α induction (38,39) and that Tax activates the NF-κB pathway (17). To
investigate the involvement of the NF-κB pathway in the Tax-induced transactivation of MIP-3α. HeLa cells were co-transfected with the MIP-3α promoter construct (pGL2-MIP-3α) and the Tax expression vector (pLTEN), together with either the expression vector of the super repressor form of IκBα (IκBαSR) or its control empty vector (pcDNA3). The Tax-dependent activation of the reporter activity was almost completely blocked in the presence of IκBαSR (Fig. 4A, left), similar to the complete repression of the control κB-Luc reporter activity by IκBαSR (Fig. 4A, right), indicating that IκBαSR suppressed the NF-κB activity caused by Tax. When JPX-9 cells were used for the experiments, a similar inhibitory effect of IκBαSR was observed (Fig. 4B). These results indicate that the Tax-mediated activation of the MIP-3α promoter is dependent on the NF-κB activity.

The NF-κB site at −82 in the MIP-3α promoter is critical for the Tax-mediated activation

In the MIP-3α promoter, we recently identified an NF-κB-binding element, at −82 bp upstream of the putative transcription start site (herein, −82-κB), which has been shown to be crucial for the response to TNF-α stimulation (38). To investigate the role of this −82-κB element in Tax responsiveness, this sequence was mutated (Fig. 5A) and this −82-κB mutant reporter construct (pGL2-MIP-3ακBM) was co-transfected with the Tax expression vector in HeLa cells. As shown in Fig. 5 (B, left), the Tax-induced promoter activity was significantly reduced in this κB mutant, indicating that Tax-transactivation of the MIP-3α promoter is dependent on the −82-κB element. When JPX-9 cells were used for the experiments, a significant reduction of the promoter activity was also observed (Fig. 5B, right). These results suggest that the −82-κB element in the promoter is critical for the Tax-induced MIP-3α gene transcription.

The NF-κB activation and the κB site at −82 are critical for the induction of MIP-3α promoter activity in HTLV-I-infected T cells

To examine the regulation of the MIP-3α promoter further, we transfected the pGL2-MIP-3α reporter into Jurkat and HUT102 cells. In Jurkat, an HTLV-I-negative T cell line, the activity of the pGL2-MIP-3α reporter was almost the same as that of the control reporter, pBL2-basic. In contrast, the activity of pGL2-MIP-3α was much higher than that of the control reporter in HUT102, an HTLV-I-infected cell line (Fig. 6A). In addition, the activity of the pGL2-MIP-3α reporter in HUT102 cells, as well as the activity of the positive control pNFKB-Luc reporter, was almost completely blocked by the co-transfection of the IκBαSR expression vector (Fig. 6B). Furthermore, the luciferase activity in HUT102 cells was markedly reduced in the −82-κB mutant reporter (pGL2-MIP-3ακXM) as compared with the wild-type reporter (Fig. 6C). These results indicate that the MIP-3α promoter is constitutively active in HTLV-I-infected cells and that the −82-κB element in the MIP-3α promoter is critical for this activation.

Binding of NF-κB/Rel family proteins to the −82-κB element in the MIP-3α promoter

We next examined whether NF-κB/Rel family members can bind to the −82-κB element by EMSA. A synthetic oligonucleotide containing the −82-κB element of the MIP-3α promoter was used as the probe. With nuclear extracts from JPX-9 cells, the induction of Tax expression led to the formation of two specific complexes with the −82-κB probe (Fig. 7, lane 2). The lower complex was competed away by competitors, such as the typical κB competitor from the IRF-1 promoter and the −82-κB oligonucleotide, but not by the mutated −82-κB oligonucleotide (Fig. 7, lanes 3–5), whereas the upper

Fig. 4. The NF-κB pathway is critical for the Tax-induced MIP-3α promoter activity. (A) HeLa cells were co-transfected with 0.5 μg of either pGL2-MIP-3α (MIP-3α) or pNFKB-Luc (κB), with 0.25 μg of either pLXEN (−) or pLTEN (Tax), and with either 0.25 μg of pcDNA3 (−) or pcDNA3-IκBαSR (IκBαSR), and 10 ng of pRL-βact. Luciferase activities were measured 48 h after transfection. The results, corresponding to the means of three independent experiments, are expressed as fold induction relative to the basal level of cells co-transfected with either pGL2-MIP3α or pNFKB-Luc and with pLXEN and pcDNA3; bars, SE. (B) JPX-9 cells were co-transfected with either 0.9 μg of pGL2-MIP3α (MIP-3α) or pNFKB-Luc (κB), and with either 1 μg of pcDNA3 (−) or pcDNA3-IκBαSR (IκBαSR), and 0.1 μg of pRL-βact. Tax induction and the measurement of luciferase activity were carried out as described in the legend to Fig. 3. The results, corresponding to the means of three independent experiments, are expressed as fold induction relative to the basal level of cells co-transfected with either pGL2-MIP3α or pNFKB-Luc and with pcDNA3, incubated in the absence of ZnCl2; bars, SE.
complex was only competed away by the $-82$-κB oligonucleotide. Furthermore, this lower complex formation was inhibited by the addition of anti-p50 or anti-p65 antibodies, but not by the anti-c-Rel antibody, suggesting that the lower complex was competed away by the $-82$-κB oligonucleotide. Furthermore, this lower complex formation was inhibited by the addition of anti-p50 or anti-p65 antibodies, but not by the anti-c-Rel antibody, suggesting that the lower complex
consistent of the p50 and p65 subunits. Because the upper complex formation was not inhibited by addition of these antibodies, this complex might unidentified protein specifically bind −82-κB. Thus, Tax induces MIP-3α gene expression, at least in part, through the induced binding of the p50 and p65 NF-κB/Rel family members to this −82-κB element.

**The CCR6 expression is increased in HTLV-I-infected cells**

We further investigated the expression of CCR6, a specific receptor for MIP-3α, in HTLV-I-positive T cells, by RT-PCR using CCR6-specific primers. HUT102 and MT-2, strongly expressed the CCR6 mRNA (Fig. 8A), whereas KK-1 and ST-1 weakly expressed it (data not shown). The HTLV-I-negative T cell lines, Jurkat and MOLT4, expressed much lower amounts of the CCR6 mRNA (Fig. 8A). When JPX-9 cells were stimulated with ZnCl2, the CCR6 mRNA was not increased, regardless of Tax induction (data not shown). The cell surface expression of CCR6 was also detected in HUT102 and MT-2 by the flow cytometric analysis (Fig. 8B), whereas marginal peak was detected in KK-1 and ST-1 (data not shown) or no positive peak was detected in Jurkat and MOLT4 (Fig. 8B).

**Discussion**

MIP-3α is expressed in inflamed tissues, such as tonsils, appendix, lymph nodes, thymus, and keratinocytes. Although it was previously reported that MIP-3α was mainly expressed in lymphoid organs (21), this chemokine is not expressed in Jurkat T cells (20). Consistent with this observation, we also could not detect MIP-3α expression in Jurkat T cells without the presence of Tax. Thus, Tax may induce MIP-3α expression in a tissue where MIP-3α is not originally found, as seen in other aberrantly expressed genes in the presence of Tax.

(40,41). There is an NF-κB-binding site (−82-κB) in the MIP-3α promoter, which is involved in the response to TNF-α (38). The fact that mutations in this κB element reduced the promoter activity suggested the crucial role of the −82-κB element in the Tax-induced transactivation of MIP-3α. Furthermore, the observations that super IκBα blocked the Tax-induced activation of the MIP-3α promoter, not only in ectopically Tax-transduced HeLa cells but also in the MIP-3α-expressing HUT102 cells and the ZnCl2-treated JPX-9 cells, allowed us to conclude that the activation of the MIP-3α gene by the HTLV-I Tax protein is primarily dependent on the NF-κB pathway. It also suggested that the transactivation by Tax occurs upstream of IκBα in the NF-κB pathway, which is consistent with the previous findings that Tax activates NF-κB by activating IκKs and other related molecules (42–48).

NF-κB might contribute to MIP-3α induction in other viral infections as well. It was reported that MIP-3α expression in monocytes is induced by Sendai virus infection, which
suggested that induced chemokines, including MIP-3α, might cause some of the pathology of the viral infections (49). Sendai virus-induced MIP-3α expression did not require de novo protein synthesis, rather it occurred in the presence of a protein synthesis inhibitor, cycloheximide, indicating some inhibitory protein(s), such as IkB, regulates the expression of MIP-3α. As Sendai virus has been reported to activate the NF-κB pathway (50), it is reasonable to speculate that the MIP-3α expression induced after the infection is, at least in part, dependent on the induced NF-κB activity.

Cells that migrate to MIP-3α are known to express its receptor, CCR6. In this study, we also identified the up-regulated expression of CCR6 in HTLV-I infected T cells. The transcriptional regulation of CCR6 gene has not been clarified yet, however, as CCR6 up-regulation was not observed in Tax-expressing Jurkat cells, the regulation of its expression seems to be independent of Tax. With immunopathological analyses, the expressions of CCR6 and MIP-3α in lesional cells were shown in psoriasis, atopic dermatitis and autoimmune encephalomyelitis (51–53). However, the concomitant expression of CCR6 and MIP-3α on the same type of cells has been reported only in pancreatic cancer cases, where MIP-3α and its receptor CCR6 were expressed within pancreatic tumors from clinical samples and within all four tested pancreatic cancer cell lines. In addition, MIP-3α stimulated the growth of one cell line and enhanced the migration of another cell line (54). Thus, it is possible that HTLV-I-infected lymphocytes with the associated expression of CCR6 and MIP-3α could proliferate or migrate in an autocrine/paracrine-dependent manner, and contribute to the pathogenesis of HTLV-I infection.

In addition to HTLV-I-infected T lymphocytes, the cells that migrate to MIP-3α, such as immature dendritic cells and lymphocytes, also should be considered in the pathogenesis of the infection. The involvement of dendritic cells in HTLV-I infection has been discussed in HAM/TSP, as the peripheral blood of HAM/TSP patients contains HTLV-I-infected dendritic cells harboring HTLV-I have the potential to secrete MIP-3α, HTLV-I carriers are at risk for deregulated, pathological inflammatory arthropathy associated with HTLV-I. Lancet 1:441.


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Abbreviations

ATL adult T cell leukemia
EMLSA electrophoretic mobility shift assay
HAM HTLV-I-associated myelopathy
HTLV human T cell leukemia virus type

IRF IFN regulatory factor
MIP macrophage inflammatory protein
TNF tumor necrosis factor
TSP tropical spastic paraparesis

References


