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Induction of cytokines in glial cells by *trans* activator of human T-cell lymphotropic virus type I

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Human T-cell lymphotropic virus type I (HTLV-I), the cause of adult T-cell leukemia, is also associated with the neurological disease, TSP/HAM (tropical spastic paraparesis/HTLV-I associated myelopathy). The HTLV-I genome encodes a protein, Tax, that *trans* activates viral and cellular gene transcription. To understand the mechanisms for the production of cytokines by HTLV-I in nervous tissue, we examined their expression in glial cells which carried the Tax-expressing vector. We demonstrated that Tax expression enhanced the production by glial cells of interleukin (IL)-1, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and transforming growth factor (TGF)β. We suggest that the excessive production of cytokines in nervous tissue may play a role in pathogenesis of TSP/HAM. Glial cells that carry the *tax* gene may provide a model useful for in vitro study of the mechanism of production of cytokines in the nervous system.

Glial cell; HTLV-I; *tax*; Cytokine; Gene activation

1. INTRODUCTION

Human T-cell lymphotropic virus type I (HTLV-I) has been identified as the infectious agent responsible for adult T-cell leukemia [1,2]. Its genome encodes a 40-kDa nuclear protein (Tax) [3] which is associated with the leukomogenesis of this disorder. Tax is a potent *trans* activator both of the transcription directed by the viral long terminal repeat, and of the transcription of specific cellular genes such as tumor necrosis factor (TNF) α [4], granulocyte-macrophage colony-stimulating factor (GM-CSF) [5], interleukin (IL) -2 [6,7], IL-3 [8], and IL-2 receptor [6,7,9]. HTLV-I is also associated with a neurological disorder such as tropical spastic paraparesis (TSP), also called HTLV-I associated myelopathy (HAM) [10,11]. Recently, we demonstrated that introduction of *tax* gene into glial cells caused expression of the major histocompatibility complex (MHC) class I antigen [12], a key molecule involved in immunorecognition. Therefore, it is possible that Tax may produce some phenotypical changes in the neural cells of TSP/HAM patients. Neural cells can produce and/or respond to a variety of cytokines which function as both neuro- and immunomodulators [13]. It has been suggested that those cytokines may play an important role in the pathogenesis of TSP/HAM, since elevated levels of IL-6 have been demonstrated in serum and cerebrospinal fluid of HAM patients [14]. Here, we show the effect of Tax on cytokine production in glial cells by *tax* gene transfection.

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Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; HAM, HTLV-I associated myelopathy; HTLV-I, human lymphotropic virus type I; IL, interleukin; MHC, major histocompatibility complex; LPS, lipopolysaccharide; PCR, polymerase chain reaction; RT, reverse transcription; TGF, transforming growth factor; TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TSP, tropical spastic paraparesis.

2. MATERIALS AND METHODS

2.1. *Plasmid*

pMTCXdb was used as an expression vector of Tax [12]. This plasmid contains the mouse metallothionein promoter as an inducible promoter.

2.2. *Cell culture and transfection*

C6 rat glial cells were cultured at 37°C in Falcon plastic culture dishes (Becton Dickinson, Lincoln Park, NJ) with Eagle's minimal essential medium containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD). One million cells were transfected with 2 μg of pMTCXdb and 0.5 μg of pSV2Neo by the Shimadzu Somatic Hybridizer I (Shimadzu, Kyoto, Japan). After cultivation for 48 h in that medium, the cells were treated with the medium supplemented with 800 μg of G418 (Gibco BRL) and grown for two weeks under continuous G418 selection. More than 100 G418-resistant colonies were pooled, propagated and used as *tax*-expressing cells, C6px. C6px were treated with or without lipopolysaccharide (LPS), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and $ZnSO_4$ for 12 h. Supernatant fractions were collected for measurement of cytokine contents. Cells were washed twice with phosphate-buffered saline and used for RNA preparation.

2.3. *RNA preparation and RT-PCR*

Total RNA was isolated by the method of Chomczynski and Sacchi

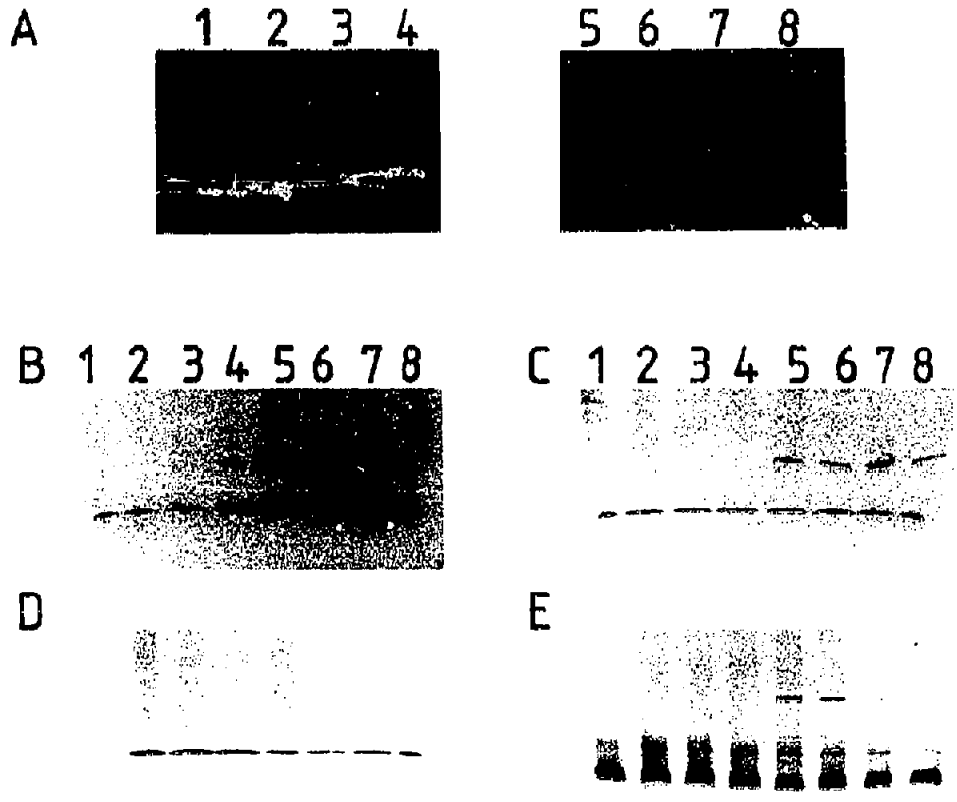

Fig. 1. mRNA expression of *tax* and cytokines in C6 (lane 1–4) and C6px (lane 5–8) cells. PCR products of *tax* (A), IL-6 (B), IL-1 (C), TNF α (D) and TGFβ (E) derived from cells treated with 100 ng/ml of LPS (lanes 2 and 6), 100 ng/ml of TPA (lanes 3 and 7), 80 μM of $ZnSO_4$ (lanes 4 and 8), or medium (lane 1 and 5) for 12 h were indicated. (A) 2% agarose gel electrophoresis. (B–E); 8–25% polyacrylamide gel electrophoresis.

[15]. An amount of 1 μg of RNA was used for first-strand cDNA synthesis at 37°C for 90 min with 50 units of recombinant M-MuLV reverse transcriptase (Boehringer-Mannheim, Mannheim, Germany) and 0.2 μg of DNA random hexamers. The reaction was terminated by boiling for 5 min, and 2 μl of the mixture was amplified by 30 PCR cycles with 0.5 units of taq polymerase (Promega, Madison, WI) and 0.5 μg each of the sense and antisense primers. The thermal cycle profile was as follows: 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. An aliquot (5 μl) of the PCR mixture was subjected to electrophoresis in 2% agarose gel or 8–25% polyacrylamide gel in TBE buffer. The agarose gel was then stained with ethidium bromide and photographed. Polyacrylamide gel was stained by the standard silver staining method. The PCR products were identified by the size which were predicted from the sequence (described in section 2.4).

2.4. *Primers for PCR*

The following primers were used for gene amplification; *tax* sense, 5'CCCACTTCCCAGGGTTTGGACGAG; *tax* anti-sense, 5'CTGTAGAGCTGAGCCGATAACGCG; IL-1β sense, 5'-GAAGCTGTGGCAGCTACCTATGTCT; IL-1β antisense, 5'-CTCTGCTTGAGAGGTGCTGATGTAC; IL-6 sense, 5'-GACTGATGTTGTTGACAGCCACTGC; IL-6 anti-sense, 5'-TAGCCACTCCTTCTGTGACTCTAACT; TNF α sense, 5'-CACCACGCTCTTCTGTCTACTGAAC; TNF α anti-sense, 5'-CCGGACTGCGTGATGTCTAAGTACT; TGF β sense, 5'-CTCCCACTCCCGTGGCTTCTAG; and TGF β anti-sense, 5'-GTTCCACATGTTGCTCCACATTG. The sizes of PCR products for *tax*, IL-1β, IL-6, TNF α, and TGF β were 203 bp, 520 bp, 509 bp, 546 bp, and 472 bp, respectively.

Table 1

Cytokine activity in medium of C6 and C6px cells

Treatment	IL-1 activity (mU/ml)		IL-6 activity (U/ml)		GM-CSF activity (U/ml)	
	C6	C6px	C6	C6px	C6	C6px
Medium	0	14	10	620	0	0.46
$ZnSO_4$ (80 M)	0	15	31	1490	ND	ND
TPA (0.1 μg/ml)	6	280	67	2350	ND	ND
LPS (0.1 μg/ml)	6	50	48	1930	0.28	0.60

One million cells were seeded in 10-cm diameter plastic dishes with 10 ml medium and treated as indicated for 12 h. ND = not determined. IL-1, interleukin-1; IL-6, interleukin-6; GM-CSF, granulocyte-macrophage colony-stimulating factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; LPS, lipopolysaccharide. Values indicate the mean of two independent experiments of each measurement carried out in triplicate. SD values were less than 10% of the mean.

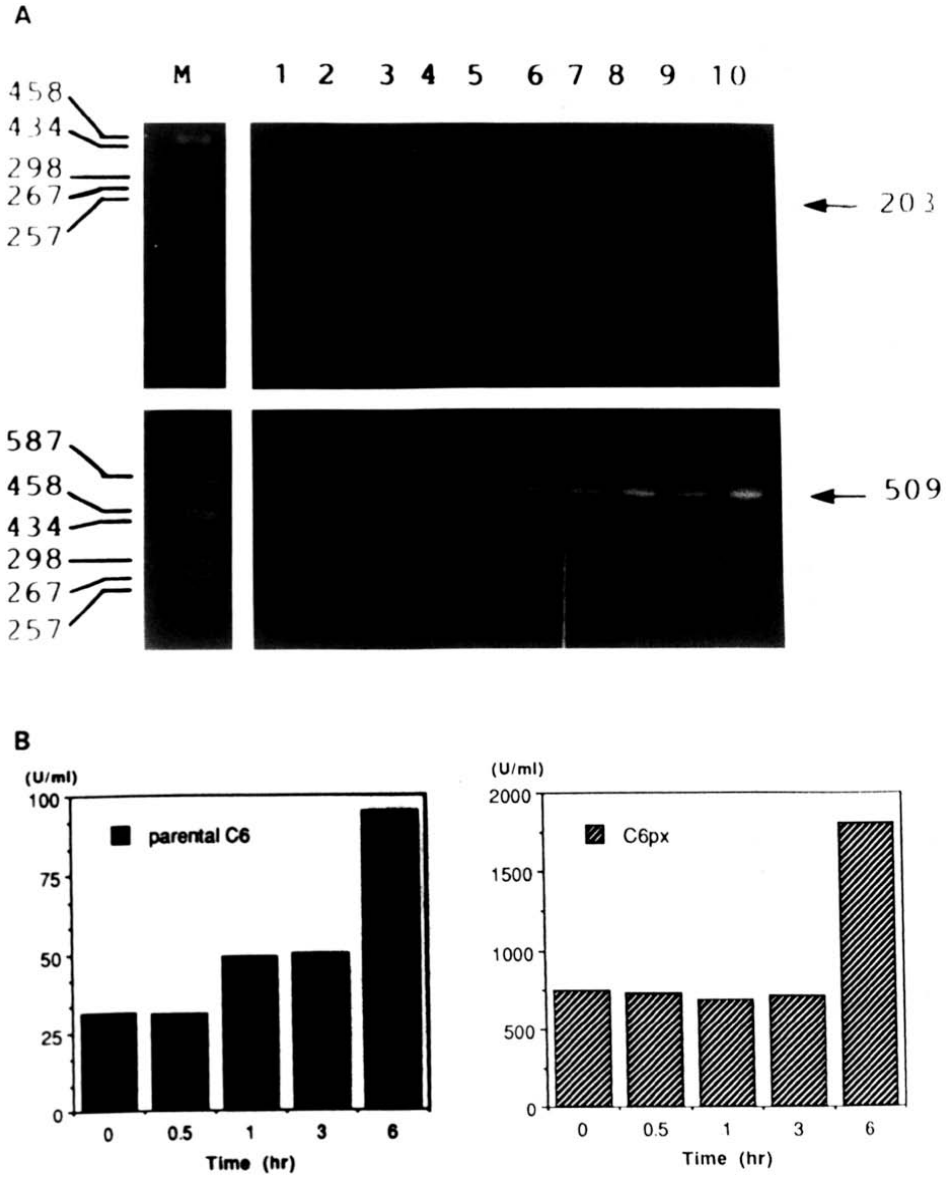

Fig. 2. (A) Time-dependent expression of *tax* (upper) and IL-6 (lower) mRNA in C6 (lanes 1–5) and C6px (lanes 6–10) cells at 0 (lanes 1 and 6), 0.5 (lanes 2 and 7), 1 (lanes 3 and 8), 3 (lanes 4 and 9) and 6 h (lanes 5 and 10) after LPS stimulation. M, size marker. (B) Time-dependent change in IL-6 activity in the supernatant of C6 (left) and C6px (right) after LPS stimulation. Columns indicate the mean of two independent experiments of each measurement carried out in triplicate SD values were less than 10% of the mean.

2.5. *Cytokine assays*

Activities of IL-1, IL-6 and GM-CSF were determined by bioassay using D10 cells (from Dr. K. Onozaki, Nagoya City University) [16], MH60 (from Dr. T. Hirano, Osaka University, Japan) [17] cells and IC2 cells (from RIKEN Cell Bank) [18], respectively. TNF α activity was measured by colorimetric assay described previously [19].

3. RESULTS

C6px, the pMTCXdb-transfected cells, expressed *tax* mRNA under normal culture conditions. Its expression was enhanced by agents that stimulated the metallothionein promoter such as LPS, TPA, and $ZnSO_4$ (Fig. 1A). Expression of *tax* mRNA was accompanied by that for IL-1, IL-6 and TGFβ (Fig. 1B, C and E). Expression of IL-6 mRNA in C6px further increased, following stimulation of metallothionein promoter with LPS, TPA, and $ZnSO_4$ (Fig. 1B). Expression of IL-1 mRNA in C6px increased when stimulated by TPA (Fig. 1C, lane 7). Expression of TGFβ was observed in C6px but not in C6 cells (Fig. 1E). Production of cytokine activities was also enhanced by *tax*-transfection (Table I). C6px increased the activity of IL-6 by 62-fold as compared with parental C6 cells. The activity of IL-1 and GM-CSF was detected in C6px. Further enhancement of IL-1, IL-6, and GM-CSF activity was observed in C6px treated with $ZnSO_4$, TPA and/or LPS. Neither TNFα mRNA (Fig. 1D) nor TNFα activity were detected.

To investigate the relationship between Tax expression and the enhancement of cellular promoters, we examined the time-dependent change of IL-6 mRNA and the biological activity in C6 and C6px following LPS treatment. The *tax* mRNA expression in C6px increased at 30 min and peaked at 60 min after LPS treatment (Fig. 2A). IL-6 mRNA expression in C6px increased at 6 h after LPS treatment (Fig. 2A). IL-6 activity in C6px also increased at 6 h after LPS treatment, although that in parental C6 cells increased gradually from 60 min to 6 h (Fig. 2B).

4. DISCUSSION

This study of C6px, the glial cells that carry the HTLV-I *tax* gene, demonstrates that Tax stimulated the production of IL-1, IL-6, GM-CSF and TGFβ in cells derived from nervous tissues. This was supported by the following lines of evidence in the case of IL-6: (i) transfection of *tax*-expression vector to C6 cells enhanced both mRNA and biological activity of IL-6 (Fig. 1 and Table I); (ii) stimulation of metallothionein promoter increased IL-6 production in C6px (Fig. 1 and Table I); and (iii) the enhancement of IL-6 production in LPS-stimulated C6px was slower than that in parental C6 cells (Fig. 2). This suggests that *tax* mRNA may be translated into Tax, followed by the induction of IL-6 mRNA in C6px by a *trans* activation of IL-6 gene by Tax. This is the first study to demonstrate that Tax induces the production of cytokines in neural cells, although similar phenomena have been demonstrated in T-cells and fibroblasts [8]. While TNF has also been demonstrated to be overproduced in T-cells of patients with HTLV-I [4], we could not detect either the mRNA (Fig. 1D) or the biological activity of TNFα. We did not find that C6px express either IL-2 or IL-2 receptor (data not shown), which are known to be expressed in T-cells carrying *tax* [6,7,9]. Therefore, it is possible that different mechanisms may control gene expression of some cytokines, but not all, in the neural cells and T-cells. Moreover, since each cytokine expression in neural cells by *tax* transfection with or without stimulants showed different patterns, it may be regulated in a different manner. Further investigations of cytokines in neural cells are necessary for understanding that.

It was recently reported that Tax enhanced the transcription of proenkephalin mRNA in glial cells [20]; enkephalin, a neuropeptide, influences the immune system. Similarly, cytokines contribute to neuro-immune system communications, but more directly than does enkephalin. As reported previously, glial cells produce a variety of cytokines which regulate both the nervous and the immune systems [13]. When Tax enhanced cytokines production in patients with HTLV-I-related diseases, it causes perturbation of the cytokine network in both the immune and nervous systems. We also reported that *tax*-transfected glial cells were induced to express the MHC class I molecule [12], which is important in recognition by T-cells. Such changes in Tax-expressing neural cells may be involved in the pathogenesis of HTLV-I-associated neurological disease. The pathological role of *trans* activation by Tax awaits further clarification.

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