Human T cell leukaemia virus type 1 p21X mRNA: constitutive expression in peripheral blood mononuclear cells of patients with adult T cell leukaemia

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Although the p21X protein of human T cell leukaemia virus type 1 (HTLV-1) is generally thought to be expressed from a doubly spliced mRNA transcript (tax/rex mRNA) that encodes the p40tax, p27rex and p21X proteins, we have shown previously that a novel, alternatively spliced mRNA transcript (p21X mRNA) is responsible for p21X production in HTLV-1-infected cell lines. In the present study, we analysed expression of p21X mRNA and tax/rex mRNA in uncultured and cultured peripheral blood mononuclear cells (PBMCs) from eight patients with adult T cell leukaemia by using a quantitative polymerase chain reaction coupled to reverse transcription. The results demonstrated that the expression of p21X mRNA occurs constitutively in all uncultured and cultured PBMCs, whereas the expression of tax/rex mRNA is inducible in the cultured PBMCs, as described previously. In uncultured and cultured PBMCs from the one specimen in which p21X mRNA was highly expressed, the p21X protein was detectable by Western blotting. On the other hand, p27rex protein was detectable only after cultivation. These findings indicate that p21X mRNA is constitutively expressed in vivo and is responsible for production of p21X protein.

Introduction

Human T cell leukaemia virus type 1 (HTLV-1) is the causative agent of adult T cell leukaemia (ATL), a malignant disorder affecting CD4+ lymphocytes (Hinuma et al., 1981; Poiesz et al., 1981; Yoshida et al., 1982). ATL is a neoplastic disease caused by the proliferation of a single cell clone, and usually one copy of the HTLV-1 genome is clonally integrated into the leukaemic cell population (Seiki et al., 1984). HTLV-1 is also aetiologically associated with a subgroup of chronic progressive myelopathies, tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM) (Gessain et al., 1985; Osame et al., 1986).

HTLV-1 is an exogenous human retrovirus. In addition to the gag, pol and env gene sequences, the HTLV-1 genome contains a unique regulatory sequence termed pX that is, postulated to be associated with leukaemogenesis in ATL (Seiki et al., 1983, 1985). The pX sequence is reported to encode p40tax, p27rex and p21X (Kiyokawa et al., 1985). p40tax is known to activate transcription of the viral genes (Lee et al., 1984; Slamon et al., 1984; Goh et al., 1985; Sodroski et al., 1984; Fujisawa et al., 1985) and some cellular genes (Inoue et al., 1986; Maruyama et al., 1987; Wano et al., 1988; Miyatake et al., 1988), and p27rex is a post-transcriptional regulator which induces the accumulation of unspliced viral gag-pol mRNA and singly spliced env mRNA (Nagashima et al., 1986; Inoue et al., 1987; Iida et al., 1987). The complete amino acid sequence of p21X is contained within the C-terminal portion of p27rex. The function of p21X is not known.

The PX sequence has been suggested to be expressed as a doubly spliced mRNA (named tax/rex mRNA) that encodes p40tax, p27rex and p21X (Yoshida, 1987). In contrast to this concept, we have recently discovered a novel, alternatively spliced mRNA capable of specifically expressing p21X (named p21X mRNA) in most HTLV-1-infected cell lines by using a highly sensitive method involving a polymerase chain reaction (PCR) coupled to reverse transcription (RT–PCR) (Orita et al., 1991).

Expression of HTLV-1 genes could not be detected by immunofluorescence analysis or RNA blot analysis in most fresh peripheral blood mononuclear cells (PBMCs) from patients with ATL (Noonan & Roninson, 1988;
Franchini et al., 1984; Hoshino et al., 1983; Sugamura et al., 1984) or asymptomatic HTLV-1 carriers (Sugamura et al., 1984). However, by applying RT–PCR it has recently been reported that tax/rex mRNA is expressed in fresh PBMCs of some ATL patients and asymptomatic carriers (Kinoshita et al., 1989). This report suggests that the expression of tax/rex mRNA as a proportion of total RNA in these PBMCs is very low, and that some viral antigens are expressed only in a small number of circulating blood cells in vivo. Furthermore, another report has shown that tax/rex mRNA expression is detectable in fresh uncultured PBMCs from seven patients with TSP/HAM (Gessain et al., 1991). This study, using in situ hybridization analysis, indicates that in vivo tax/rex mRNA expression occurs at a high level in a few cells (1 in 5000 PBMCs). Analysis of pX mRNA expression in vivo thus seems to provide some important information on the mechanism of leukamogenesis of ATL, the pathogenesis of TSP/HAM, persistent virus infection and activation from the latent state.

In this paper, we report the nature of the p21X mRNA and tax/rex mRNA expression in PBMCs from patients with ATL using RT–PCR or a highly sensitive detection procedure of modified RT–PCR coupled to a double PCR with nested primers. Our studies also focus on analysing the mechanism of expression of p21X in order to understand its function in vivo. We detected p21X mRNA in both fresh uncultured and cultured PBMCs from all of eight patients with ATL. The constitutive expression of p21X mRNA suggests that the regulatory mechanism for p21X mRNA expression is quite different from that for expression of tax/rex mRNA.

Methods

Cells. Human T cell lines MT-2 (HTLV-1-positive) and Molt-4 (negative) were cultured in RPMI 1640 supplemented with 10% heat-inactivated foetal bovine serum. The MT-2 cell line was originally established by cocultivation of human cord blood lymphocytes from normal subjects with PBMCs from an ATL patient (Miyoshi et al., 1981). The Molt-4 cell line was established from a patient with acute lymphoblastic leukaemia (ATCC CRL-1582). Heparinized peripheral blood samples were obtained from patients with ATL, six of the acute type and two of the chronic type (Table 1). PBMCs were isolated by ficoll-paque (Pharmacia). After isolation, the cells were cultured in the same medium supplemented with recombinant interleukin 2 (IL-2; final concentration 100 units/ml; Shionogi).

Synthetic oligonucleotides and other reagents. Oligonucleotide primers for PCR were synthesized on a cyclone DNA synthesizer (Biosearch) using the phosphoramidite method, and purified with oligonucleotide purification cartridges (Applied Biosystems). A random hexaDNA nucleotide primer, T4 polynucleotide kinase, avian myeloblastosis virus (AMV) reverse transcriptase and Therma aquaticus (TAG) DNA polymerase were purchased from Takara Shuzoh.

RNA preparation. RNA was isolated from cell lines or PBMCs by the guanidinium thiocyanate–phenol–chloroform method as described previously (Chomczynski & Sacchi, 1987). The amount and quality of RNA were estimated by measuring A260 and A280, and by comparing the relative intensities of the bands of 18S and 28S rRNAs visualized on a formaldehyde–agarose gel by staining with ethidium bromide.

Quantitative RT/two step PCR (RT/TS-PCR). RT/TS-PCR was carried out as described previously (Orita et al., 1991). Briefly, 0.5 μg of total RNA was annealed with 500 ng of a random hexadecanucleotide primer, reverse-transcribed using 15 units of AMV reverse transcriptase at 42 °C for 1 h, and 8.3 μl of 6 × PCR buffer (1 × PCR buffer consists of 16.6 mM-ammonium sulphate, 67 mM-Tris-HCl pH 8.8 at 25 °C, 6.7 mM-magnesium chloride, 10 mM-2-mercaptoethanol, 7.7 μM-EDTA and 170 μg/ml BSA), 5 μl each of 15 mM-dATP, 15 mM-dCTP, 15 mM-dGTP and 15 mM-dTTP, and 1 pmol of the PCR primers PX1 and PX2 were then added to the reaction vessel to give a final volume of 50 μl. The sequences of the primers for PCR (PX1, PX2, PX3, PX4 and PX10) are shown in Fig. 1. PX1 and PX2 are located upstream and downstream, respectively, of the splice junction site of p21X mRNA.

(a) Genomic/gag-pol env poly(A)

(b) Primer

Table 1. Clinical types and haematological features of patients with ATL

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>(Age/Sex)*</th>
<th>Diagnosis</th>
<th>Aberrant cells (%)</th>
<th>WBC* (×10³ per mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(64/f)</td>
<td>Chronic</td>
<td>44</td>
<td>15.4</td>
</tr>
<tr>
<td>2</td>
<td>(82/m)</td>
<td>Acute</td>
<td>75</td>
<td>11.6</td>
</tr>
<tr>
<td>3</td>
<td>(78/f)</td>
<td>Acute</td>
<td>89</td>
<td>1.9</td>
</tr>
<tr>
<td>4</td>
<td>(64/f)</td>
<td>Acute</td>
<td>22</td>
<td>14.7</td>
</tr>
<tr>
<td>5</td>
<td>(36/m)</td>
<td>Acute</td>
<td>37</td>
<td>20.0</td>
</tr>
<tr>
<td>6</td>
<td>(70/m)</td>
<td>Acute</td>
<td>22</td>
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<td>7</td>
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<td>Acute</td>
<td>60</td>
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<tr>
<td>8</td>
<td>(72/f)</td>
<td>Chronic</td>
<td>50</td>
<td>13.0</td>
</tr>
</tbody>
</table>

* Patient numbers correspond to the sample numbers in Fig. 2(b) and 3(a).

† WBC, White blood cells.
The first-step PCR was then carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus Instruments) for 25 cycles with 2·5 units of Taq polymerase. The reaction conditions were 1 min at 94°C for denaturation, 1 min at 55°C for primer annealing and 2 min at 72°C for primer extension. One-tenth of the first-step PCR DNA product was used as template DNA for the second-step PCR with 100 pmol of each primer pair (PX10 and PX4 for targeting p21X mRNA, and PX3 and PX4 for tax/rex mRNA) for 35 cycles. The other reaction conditions were as above. PX10 and PX4 are located upstream and downstream, respectively, of the specific junction site of p21X-specific mRNA. PX3 and PX4 are located upstream and downstream, respectively, of the second splice junction site of tax/rex mRNA. After the reaction, one-fifth of the amplified DNA product was electrophoresed in a composite gel of 2% Nusieve agarose and 1% standard agarose (FMC BioProducts), and stained with ethidium bromide (0·5 µg/ml). The presence of a band of the expected size for each primer pair was judged as a positive reaction. The final amplified DNA fragments after RT/TS-PCR were expected to be 272 bp for tax/rex mRNA and 81 bp for p21X mRNA when amplified with primers PX10 and PX4, and 144 bp for tax/rex mRNA when amplified with primers PX3 and PX4. The specificity of these amplified products was confirmed by hybridization with the specific probes as described previously (Orita et al., 1991).

Detection of p27 rex and p21X proteins by Western blot analysis. Western blot analysis of p27 rex and p21X was performed as described previously (Sato et al., 1990) with modifications. Briefly, proteins were extracted from cells, separated by 18% SDS-PAGE and transferred to a membrane (Clear Blot membrane-P; ATTO). Blotted proteins were incubated with a rabbit antibody against the C-terminal peptide (TSFPPPSPGPSCP-T-COOH, which reacts with both p27 rex and p21X) conjugated Protein A and enhanced chemiluminescence (ECL) substrate solution (Amersham).

Results

Demonstration of p21X mRNA and p21X protein expression in fresh uncultured PBMCs of patients with ATL

In our recent report (Orita et al., 1991), RNA from HTLV-1-infected cell lines was analysed to evaluate the sensitivity and specificity of RT/TS-PCR. The results indicate that the sensitivity of RT/TS-PCR is about 103- to 104-fold higher than that of the conventional RT-PCR procedure, and that the specificity is sufficient without using hybridization with radioisotope-labelled probes. As shown in Fig. 2(a), RNA from MT-2 cells, which contain multiple copies of HTLV-1 provirus and produce a large amount of virus, was analysed by RT/TS-PCR. After RT/TS-PCR, the specific signals, a 144 bp band, could be detected at dilutions from < 1- to 102-fold. The data indicate that the amount of tax/rex mRNA in fresh uncultured PBMCs from patients with ATL by RT–PCR (Kinoshita et al., 1989). As shown in Fig. 2(b), we could also observe low-level tax/rex mRNA expression in fresh uncultured PBMCs in most cases. In patient 4, tax/rex mRNA could not be detected even in undiluted RNA preparations. Collectively, tax/rex mRNA in the fresh uncultured PBMC RNA could be detected at dilutions from < 1- to 104-fold. The data indicate that the amount of tax/rex mRNA in the fresh uncultured PBMCs of ATL patients is > 105-fold less than that in MT-2 cells. In the same fresh uncultured PBMCs, we could detect the same p21X mRNA-specific 81 bp band as in MT-2 cells (Orita et al., 1991). The 81 bp bands could be detected at dilutions from 101- to 106-fold in the eight specimens (Fig. 2b, Table 2). The data indicate that...

Table 2. Quantitative analyses for expression of viral transcripts tax/rex mRNA and p21X mRNA, and viral proteins p27 rex and p21X in PBMCs from eight patients with ATL

<table>
<thead>
<tr>
<th>Patient</th>
<th>tax/rex</th>
<th>p21X</th>
<th>p27 rex</th>
<th>p21X</th>
</tr>
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<tr>
<td>1</td>
<td>10⁴</td>
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<td></td>
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<td>10⁴</td>
<td>10⁴</td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>10²</td>
<td>10⁴</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>&lt;10⁶</td>
<td>10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10²</td>
<td>10³</td>
<td></td>
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<tr>
<td>6</td>
<td>10⁴</td>
<td>10³</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>10⁴</td>
<td>10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>10²</td>
<td>10³</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Amounts of mRNA were quantitatively estimated as described in the text. RNA was titrated in 10-fold dilutions for RT/TS-PCR and the reciprocal of the endpoint dilution that gave a positive reaction is indicated.
† Amounts of expressed proteins were determined by semiquantitative estimation by comparison of the intensity of immunoblotting bands with that of the most abundantly expressing cells, and were categorized into four groups: -, no band visible; +, < 10% of the strongest; ++, > 10% of the strongest; ++++, the strongest.
¶ The patient numbers correspond to those given in Table 1.
§ -, Uncultured PBMCs; +, cultured PBMCs.
p21X mRNA is expressed in all eight PBMC samples, and the amount of p21X mRNA detected corresponded to 10²- to 10⁴-fold less than that in MT-2 cells.

Western blot analysis using antibody against the C-terminal oligopeptide of p27rex/p21X was carried out (Fig. 3). The p27rex- and p21X-specific bands detected in MT-2 cells served as a positive control. In none of the fresh uncultured PBMCs was p27rex detected because the level of tax/rex mRNA expression was so low that the amount of p27rex expressed from the mRNA was not high enough for detection by Western blot (Fig. 3, Table 2). In contrast, p21X could be detected in one specimen (patient 3) which also contained the highest amount of p21X mRNA, as already mentioned. The specificity of this reaction was confirmed by blocking with the specific oligopeptide (Fig. 3b). However, no p21X was detected in the other specimens probably because the amount of p21X mRNA in them was at least 10²-fold less than that of patient 3. The data suggest that p21X mRNA, but not tax/rex mRNA, is responsible for expressing p21X in PBMCs from patients with ATL as well as in HTLV-1-infected cell lines (Orita et al., 1991).

No induction of p21X mRNA and p21X protein in cultured PBMCs from ATL patients despite high induction of tax/rex mRNA and p27rex

It is well known that tax/rex mRNA is rapidly induced after in vitro cultivation of fresh PBMCs from patients with ATL (Kiyokawa et al., 1985), and it has been
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(a) Total cell extracts from fresh uncultured PBMCs (−), PBMC cultured for 3 days (+) and the HTLV-1-infected cell line MT-2 were separated by SDS-PAGE and transferred to a filter. Blotted proteins were incubated with the rabbit antibody against p27rex and p21X. The immune complexes were bound with horseradish peroxidase-conjugated protein A, and detected using an ECL detection system (Amersham). (b) The total cell extract of fresh uncultured PBMCs from ATL patient 3 was analysed by Western blotting-ECL using the rabbit antibody against p21X/p27rex, unabsorbed (lane 1) and preabsorbed with the C terminus oligopeptide of p21X/p27rex (10 µg/ml, lane 2; 100 µg/ml, lane 3) or with the N terminus oligopeptide of p40tax (MAHFPFGQSSLFGYPVVVFDCVCOOH; 10 µg/ml, lane 4; 100 µg/ml, lane 5), serving as a negative control. The MrS of the markers (prestained SDS-PAGE standards, low range; Bio-Rad) are indicated to the left.

Discussion

The pX sequence of HTLV-1 was thought to be expressed as a doubly spliced mRNA transcript (tax/rex mRNA) encoding p40tax, p27rex and p21X, although the possibility that p27rex and p21X are independent translation products from differently spliced mRNAs remained (Kiyokawa et al., 1985). As reported recently, we have examined the latter possibility and demonstrated that p21X is translated from a novel, alternatively spliced mRNA, p21X mRNA, in HTLV-1-infected cell lines (Orita et al., 1991). Since p21X mRNA is detected in most HTLV-1-infected cell lines and contains only the first and third exons of tax/rex mRNA, it can encode only p21X. The quantitative level of p21X mRNA expression correlates with the amount of p21X expressed in cells.

Therefore, in the present study, we examined whether p21X mRNA could also be expressed in vivo. To this end, we examined pX mRNA expression in fresh uncultured and cultured PBMCs from patients with ATL by using RT/TS-PCR. It is well known that leukaemia or lymphoma cells from all ATL cases tested contain the HTLV-I proviral genome and that the primary tumour cells of all cases are monoclonal (Seiki et al., 1984; Yoshida & Seiki, 1987). Therefore, it could reasonably be assumed that a great number of PBMCs from ATL patients in this study (Table 1) are monoclonal leukaemic cells derived from HTLV-1 infection. In fresh uncultured PBMCs from all eight patients with ATL, no or low level tax/rex mRNA expression was observed, whereas p21X reported that cultured cells express p40tax and p27rex, but not p21X. We confirmed this observation by examining tax/rex mRNA expression in cultivated PBMCs from eight patients with ATL. After a 3-day cultivation, induction of tax/rex mRNA was observed in all specimens tested, and in most the induction was very high (increasing >10²-fold in specimens from patients, 1, 2, 3, 5, 6 and 8) (Fig. 2, Table 2). The cultured cells expressed such high tax/rex mRNA levels that the band specific for p27rex was detected in most of the specimens by Western blot analysis (Fig. 3, Table 2). On the other hand, in the same experiment, no induction of p21X mRNA was observed after culture, i.e. the amount of p21X mRNA did not change significantly in fresh uncultured and cultured cells (Fig. 2, Table 2). The amounts of p21X expressed probably also did not change in fresh uncultured and cultured cells from patient 3 (Fig. 3). These results demonstrate that no induction of the p21X mRNA and p21X protein occurred in cultured PBMCs of ATL patients, in contrast to the extensive induction of tax/rex mRNA and p27rex. Since these results also indicated that the amount of p21X in the fresh uncultured and cultivated cells tends to be correlated with the level of p21X mRNA expression, but not with that of tax/rex mRNA expression, the data confirmed that p21X mRNA but not tax/rex mRNA is responsible for the expression of p21X in the PBMCs from patients with ATL, as in HTLV-1-infected cell lines (Orita et al., 1991). Thus, it is concluded that p21X mRNA is constitutively expressed in both fresh uncultured and cultured PBMCs from patients with ATL.
mRNA expression was observed in all the PBMC specimens tested. In addition, a preliminary study using RT/TS-PCR showed that the p21X mRNA was detected in fresh uncultured PBMCs from asymptomatic HTLV-1 carriers (data not shown). Moreover, after RT/TS-PCR of RNA preparations from the PBMCs from patients with ATL, we observed an additional amplified band of about 200 bp (Fig. 2) which was identified previously as being derived from a different, alternatively spliced viral mRNA in HTLV-1-positive cell lines (Orita et al., 1991).

The expression of low levels of tax/rex mRNA in fresh uncultured PBMCs from patients with ATL has been reported by Kinoshita et al. (1989). In this study, tax/rex mRNA was detected in most ATL patients (seven of eight; Table 2), indicating that the sensitivity of mRNA detection by our procedure was comparable to that of Kinoshita et al. The amount of tax/rex mRNA detected in each blood specimen corresponded to $10^3$- to $10^5$-fold less than that in the HTLV-1-positive MT-2 cell line. In none of them was p27rex detected by the Western blot technique. In the same specimens, the amount of p21X mRNA corresponded to $10^2$- to $10^5$-fold less than that in MT-2 cells. In the specimen from patient 3, which expressed the highest level of p21X mRNA, p21X protein was detectable by Western blotting. Thus, p21X mRNA was highly expressed in fresh uncultured PBMCs from most patients with ATL. The level of mRNA expression appears to have no clear correlation with the clinical type of disease, such as acute or chronic, or the proportion of aberrant (ATL) cells per PBMC.

In most of the PBMC specimens cultured for 3 days with IL-2, the induction of tax/rex mRNA was observed at extraordinarily high levels. In the same manner, a large amount of p27rex was induced in most samples. The variation in induction cannot be explained by the clinical condition of the donor, although it may be explained by the observation that PBMCs isolated from some, but not all, ATL patients proliferate in response to IL-2, through the IL-2 receptor constitutively expressed on the surface of leukaemic cells (Uchiyama et al., 1985, 1988). However, the question why PBMCs from some ATL patients do not proliferate in response to IL-2 remains to be answered. On the other hand, it is clear that the level of p21X mRNA expression in cultured PBMCs did not change when compared to that in uncultured PBMCs. In the case of the p21X protein from patient 3, there was also no change. From these data we can conclude that p21X is translated from p21X mRNA in leukaemic cells circulating in the peripheral blood of patients with ATL, as well as in HTLV-1 cell lines, as previously described (Orita et al., 1991). The constitutive and non-inductive expression of p21X mRNA indicates that the regulatory mechanism for expressing p21X mRNA is quite different from that for expressing tax/rex mRNA, env mRNA and genomic/gag-pol mRNA. However, details of the mechanism remain to be analysed.

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References


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